

**Pharmacokinetics and tissue withdrawal study of tulathromycin  
in North American bison (*Bison bison*) and  
white-tailed deer (*Odocoileus virginianus*)  
using liquid chromatography-mass spectrometry**

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By  
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## ABSTRACT

Tulathromycin is a macrolide antibiotic approved for use in cattle and swine respiratory disease. Extra-label use of tulathromycin occurs in bison and deer and significant interspecies differences in pharmacokinetics warrant specific investigation in these species. This study involved investigation of the pharmacokinetics of tulathromycin in bison and white-tailed deer following a single 2.5 mg/kg bw subcutaneous injection (n=10) of *Draxxin* (Pfizer Inc.) to provide important information regarding tulathromycin dosage regimens in these species. As well, tulathromycin distribution and depletion in deer muscle and lung tissues following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* was investigated to obtain pilot information regarding withdrawal time of tulathromycin in deer.

For the pharmacokinetic studies, serial blood samples were collected at baseline and up to 25 days post-injection. Pharmacokinetic parameters were estimated using non-compartmental methods. For the tissue pilot study, deer (n = 2 to 3) were slaughtered at 0, 1, 2, 6, 7, and 8 weeks post-injection. A quantitative analytical liquid chromatography-mass spectrometry method for measuring tulathromycin was developed and validated in bison and deer serum and deer lung and muscle according to international guidelines. Samples were processed by solid-phase extraction. Reverse-phase chromatography was performed by gradient elution. Positive electrospray ionization was used to detect the double charged ion  $[M+2H]^{+2}$  at  $m/z$  403.9 and monitored in selected ion monitoring mode.

Tulathromycin demonstrated early maximal serum concentrations, extensive distribution, and slow elimination characteristics in deer and bison. In bison, mean  $C_{max}$  (195 ng/mL) was lower compared to cattle (300 to 500 ng/mL) and half-life (214 hours) longer (cattle, 90 to 110 hours). In deer, mean  $C_{max}$  (359 ng/mL) is comparable to cattle, but half-life (281 hours) was much longer. Tissue distribution and clinical efficacy studies are needed in bison to confirm extensive distribution of tulathromycin into lung and the appropriate dosage regimen. Tulathromycin was extensively distributed to deer lung and muscle, with tissue levels peaking within 7 to 14 days after injection. Drug tissue concentrations were detected 56 days after treatment, longer than the established withdrawal time of 44 days in cattle. This prolonged drug concentration in the tissue is supportive for the administration of tulathromycin as a single injection therapy for treatment of respiratory disease of deer. While more study is needed to

establish a recommended withdrawal time, the long serum and tissue drug half-life and extensive interindividual variability in tissue levels suggests a withdrawal period well beyond 56 days may be required in deer.

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## **DEDICATION**

To Mom and Dad.

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## LIST OF ABBREVIATIONS

ACN	Acetonitrile
AMR	Antimicrobial resistance
AUC	Area under the curve
BRD	Bovine respiratory disease
bw	Body weight
CFIA	Canadian Food Inspection Agency
CgFARAD	Canadian Global Food Animal Residue Avoidance Database
Cl/F	Apparent clearance
C <sub>max</sub>	Maximal plasma concentration
DIN	Drug Identification Number
ELDU	Extra-label drug use
EA	Ethyl acetate
ESI	Electrospray ionization
FARAD	Food Animal Residue Avoidance Databank
FDA	(U.S.) Food and Drug Administration
HPLC	High performance liquid chromatography
HQC	High quality control
k	Log-linear terminal rate constant
K <sub>2</sub> HPO <sub>4</sub>	Potassium phosphate dibasic anhydrous buffer
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
LQC	Low quality control
MeOH	Methanol
MIC	Minimum inhibitory concentration
MR	Marker residue
MRT	Mean residence time
MRL	Maximum residue limit
MUADP	Minor Use Animal Drug Program
MUMS	Minor Use and Minor Species
MQC	Middle quality control
<i>m/z</i>	Mass-to-charge ratio
PD	Pharmacodynamics
PK	Pharmacokinetics
ppm	Parts per million
QC	Quality control
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRD	Swine respiratory disease
TFA	Trifluoroacetic acid
t <sub>max</sub>	Time to maximal plasma concentration
t <sub>1/2</sub>	Half-life
VDD	Veterinary Drugs Directorate
V <sub>d</sub> /F	Apparent volume of distribution following an extravascular administration

## 1. INTRODUCTION

Limited available information regarding drug dosing and withdrawal times for bison and white-tailed deer species makes treating illnesses of any kind a challenging task for veterinarians and producers. Currently, there are no antimicrobial drugs registered in North America for use in these species, and consequently no food safety related residue limits are attached to their use in bison or deer (Woodbury, 2012). Bison and deer are typically treated with antibiotics approved for use in cattle and used at the recommended cattle dose. This practice, known as off-label or extra-label drug use, is based on the assumption that deer and bison share similar absorption and disposition characteristics as cattle. However, species differences do exist, and pharmacokinetic information obtained in one species should not be assumed to be the same in another species (Toutain et al., 2010). Significant differences between species leads to markedly different peak and duration of drug levels in different species. This means that withdrawal times and dosage regimens in the species for which the drug is licensed may be very different from bison or deer. Significant animal health and food safety risks arise with off-label drug use.

Bison and white-tailed deer industries continue to prosper in today's economy (NBA, 2013; NADeFA, 2013; AWMDA, 2011). With production intensification, diseases, including respiratory infections are on the rise in deer species (Dyer et al., 2004) and are becoming a leading cause of death in bison (Janardhan et al., 2010; Dyer et al., 2008). Tulathromycin is a macrolide antibiotic approved for use in treating bovine and swine bacterial respiratory disease (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Studies have demonstrated tulathromycin is highly effective for the treatment and prevention of bovine respiratory disease in cattle when administered as a single dose (Evans, 2005; Pfizer Inc, 2005). High efficacy of tulathromycin has also been observed in swine respiratory disease (Pfizer Inc., 2005). In these species, tulathromycin is rapidly absorbed with maximal plasma concentrations reached within 1 hour after dosing of *Draxxin* (APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Tulathromycin is widely and rapidly distributed to tissues, with accumulation occurring within the lungs (APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Elimination is slow and half-lives are between 3-5 days (Drugs.com, 2013; Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Gáler

et al., 2004). Typically, multiple injections of macrolides, such as erythromycin and tylosin are needed to reach the desired therapeutic effect (Wang et al., 2012; Young et al., 2010; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004). However, tulathromycin is formulated as a long-acting, single-dose injection therapy (APVMA, 2007; Evans, 2005; Gáler et al., 2004; Nowakowski et al., 2004; Benchaoui et al., 2004). Its chemical structure consists of three amine groups, which allows for greater penetration into the lung, and thus continued activity against the targeted bacteria after a single injection (APVMA, 2007; Evans, 2005; FDA/CVM, 2004; Gáler et al., 2004). The difficulty associated with bison and deer handling makes tulathromycin an appealing treatment option for veterinarians and producers of these animals. Today, tulathromycin is used off-label in bison and deer species. Studies are needed to investigate tulathromycin efficacy and safety in bison and deer in order to effectively treat disease and ensure food safety.

The primary goals of this thesis research were to investigate the pharmacokinetics of tulathromycin in bison and white-tailed deer, determine whether the drug reaches therapeutic concentrations in lung tissues, and to investigate tulathromycin distribution and depletion in other tissues. The pharmacokinetic studies were carried out in both bison and deer; however, due to time constraints and experimental design concerns, only the deer tissue pilot study was done for this thesis research. These objectives required the development and validation of a sensitive, specific, and accurate analytical method for quantification of tulathromycin in bison and white-tailed deer serum and selected tissues in deer. Liquid chromatography coupled to mass spectrometry (LC-MS) has become standard practice in pharmacokinetic analysis (Mulvana, 2010; Berna et al., 2004). Mass spectrometry techniques are powerful tools for the analysis of drugs in food and biological samples due to excellent sensitivity, specificity, ability to identify unknown compounds, and the ability to quantify drugs at trace levels (Mohamed et al., 2011; Wang, 2009; McGlinchey et al., 2008; Chico et al., 2008). Using LC-MS, pharmacokinetic data for tulathromycin in bison and white-tailed deer can provide information that is important for establishing bison and deer-specific tulathromycin dosage regimens. Furthermore, tissue distribution and depletion data of tulathromycin in bison and white-tailed deer provide information on appropriate drug withdrawal times, which assures consumer safety.

## **2. LITERATURE REVIEW**

### **2.1 Veterinary Drug Use in Minor Species**

Bison and deer are considered **minor species** – all animals that are not one of the major species (i.e. cattle, swine, chickens, turkeys, horses, dogs, and cats) (FDA, 2013; MUADP-1, 2013; MUADP, 2009; FDA/HHS, 2007). In general, minor species are non-traditional food-producing animals, as well as pets and zoo animals. Veterinary drug use in minor species can be challenging for a number of reasons. Very few drugs are approved for use in minor species. Research required for drug approvals is very expensive and populations of minor species are small, leaving little economic incentive for drug companies to invest in the approval process for drug use in minor species (MUADP, 2009; FDA/HHS, 2007). Unapproved drug use occurs in minor species food-producing animals (MUADP, 2009; Health Canada, 2002), which may be necessary to avoid animal suffering, but may also lead to potential issues for humans eating food products derived from animals treated with the unapproved drug (Grignon-Boutet et al., 2008; Health Canada, 2002). To help with these issues, programs and policies have been put in place to overcome and evaluate the challenges of using drugs in minor species. These issues and policies are discussed below.

#### **2.1.1 Shortage of Drugs for Minor Species**

Very few approved drugs are available for the prevention or treatment of disease in minor species (MUADP-2, 2013; MUADP, 2009; FDA/HHS, 2007). Most minor species food animals have fewer numbers of safe and effective drugs for use, unlike those available for cattle, swine, and poultry (MUADP, 2009). This problem is well recognized by veterinarians, animal producers, scientists, and regulators (MUADP-2, 2013; MUADP, 2009). Without appropriate drugs to prevent or treat disease in food animals, potential outcomes include increase in animal mortality, as well as the increase in cost of producing animal food products (MUADP, 2009). The shortage of available drugs for controlling diseases in these animals is a serious management and economic problem for producers of minor species (MUADP, 2009; FDA/HHS, 2007).



### **2.1.2 No Economic Incentive for Drug Research in Minor Species**

Pharmaceutical companies have little economic incentive to invest into the necessary research for the drug approval process in minor species (MUADP-1, 2013; MUADP, 2009; FDA/HHS, 2007). Most minor species populations are too small to justify research expenditures that demonstrate safety and efficacy (MUADP, 2009; FDA/HHS, 2007; Cattet, 2003). The process of generating the data necessary for a drug approval is very costly and time-consuming (MUADP, 2009; Cattet, 2003). The estimated cost to a pharmaceutical company for a new drug for human use exceeds one billion Canadian dollars, and takes on average 14 years to reach the marketplace (Tufts Centre, 2013). Costs for animal drugs are estimated to be over \$100 million (2008 estimate) per drug approval and require 7-10 years of research (MUADP, 2009). The cost of a new label claim is an additional \$10-25 million (MUADP, 2009). Therefore, pharmaceutical companies tend to invest in those drugs that will have reasonable potential for profit (MUADP, 2009; Cattet, 2003). Most drug approvals are pursued only for those animal species produced in sufficient numbers to support sales, specifically the major species (MUADP, 2009). As a result of this issue, the Minor Use Minor Species (MUMS) Act was legislated to provide incentives for pharmaceutical companies or sponsors to develop new animal drugs for minor species (See **2.1.5**).

### **2.1.3 Unapproved Drug Use in Minor Species**

Veterinarians and livestock producers can use unapproved (unlicensed) drugs in minor species without previous testing in these species (MUADP, 2009; Health Canada-1, 2004; Health Canada, 2002). An unapproved drug is one that does not have a valid Drug Identification Number (DIN), and whose sale has not been permitted in Canada. A **DIN** is the number on a drug product that is issued to each product and has been reviewed and approved by Health Canada. (Health Canada-2, 2009; Health Canada-1, 2004). Unapproved drug use often occurs in minor species because few approved products are available to treat a specific infection or condition (Health Canada-1, 2009; Health Canada-1, 2004). Some drugs may be recognized to be highly effective in the treatment and management of disease for which approval has not been sought in a particular species (Health Canada-1, 2009). While this may be necessary for animal welfare,

using unapproved drugs in food animals may be associated with potential animal and human health implications (Health Canada-1, 2004; Health Canada, 2003; Health Canada, 2002).

Unapproved drug use can be harmful to the animals being treated because it is not known if the drug is safe or effective for that particular species (MUADP, 2009). Animal safety could be at risk since these products usually have not been tested appropriately for these new conditions of use (Health Canada-1, 2009; Health Canada-2, 2009; MUADP, 2009) and drugs used in this manner may have reduced or uncertain efficacy for the condition(s) being treated (Health Canada-2, 2009). Unknown efficacy is a concern when a product is used in a different species, used for a different disease, or by a different route of administration than what has been approved on the label (Merck Inc., 2011; Health Canada-1, 2009). Use of drugs at dosing levels approved for a different species may also result in significant morbidity and mortality in minor species. Species differences in drug pharmacokinetics do exist and dosage regimens in the species for which the drug is licensed may be very different to those it is not approved for (Toutain et al., 2010). Veterinarians may practice extra-label drug use based on limited information from scientific evidence, research reports, or from pharmaceutical companies. They may not have the information on pharmacological principles or pharmacokinetic differences between species to use a drug safely and effectively in an unapproved manner (Health Canada-1, 2009).

Unapproved drug use can also lead to the persistence of drug residues in animal products intended for human consumption (Health Canada-2, 2009; MUADP, 2009; Craigmill et al., 2004), which poses a significant food safety risk. When food-producing animals are treated with unapproved drugs, residues may be present in food products and some level of risk may be passed on unknowingly to consumers (Health Canada-1, 2004; Wang, 2009). These residues in food products may also be a violation set under the Canadian *Food and Drug Regulations* (See **2.2.1**) (Health Canada-1, 2004; Health Canada-2, 2009). Unapproved drug use may present ethical or liability issues for veterinarians, and can lead to trade restrictions (Health Canada-1, 2009; Health Canada-2, 2009; Wang, 2009). More information regarding drug residues is presented in Section **2.2.2**.

#### **2.1.4 Minor Use Animal Drug Program (MUADP)**

The Minor Use Animal Drug Program (MUADP), formerly the National Research Support Project #7 (NRSP-7), is a United States agricultural program that addresses the shortage of, and aims to approve veterinary drugs for minor species (MUADP-1, 2013; MUADP-2, 2013). The program promotes minor species drug studies by providing funding for research through research grants and support from pharmaceutical companies, universities, and other governing agencies (MUADP-2, 2013; Craigmill et al., 2004). Efficacy, animal safety, human food safety research, and environmental assessments are also evaluated and monitored through MUADP, as is required for drug approvals (MUADP-2, 2013). The program's objectives are: (1) to identify animal drug needs for minor species; (2) to generate and provide information about safe and effective drug doses (in minor species); and (3) to facilitate the Food and Drug Administration's Centre for Veterinary Medicine (FDA/CVM) drug approvals for minor species (MUADP-2, 2013; MUADP, 2009). The primary goal of the MUADP is to increase availability of approved therapeutic drugs for minor species (MUADP-2, 2013; Craigmill et al., 2004). To accomplish these goals, the program functions through a collaborative effort among animal producers, pharmaceutical companies, government agencies, universities, and veterinary schools (MUADP-2, 2013; MUADP, 2009). Special workshops or forums are also held where issues of disease problems and drug priorities are discussed (MUADP-2, 2013). The MUADP is largely the only organized federal (U.S.) attempt to address the insufficient number of FDA approved drugs for minor species and is responsible for the majority of progress made in drug approvals for minor species (MUADP-1, 2013). There is no similar program in Canada.

#### **2.1.5 Minor Use Minor Species (MUMS) Animal Health Act**

The Minor Use and Minor Species Animal Health Act, commonly referred to as the MUMS Act, was signed into U.S. law in 2004 (FDA, 2013; FDA/HHS, 2007). This Act was designed to encourage the development of animal drugs that are currently unavailable to minor species (FDA, 2013; MUADP-1, 2013; FDA/HHS, 2007) and is intended to make more drugs legally available to veterinarians and animal owners to treat minor species (FDA, 2013; Health Canada-1, 2009; FDA/HHS, 2007). The law provides innovative ways to bring products of these small (minor species) populations to market and is designed to help pharmaceutical companies

overcome the financial barriers faced in providing limited-demand animal drugs, while still maintaining animal and public (human) health (FDA/HHS, 2007; MUADP, 2009; Health Canada-1, 2009). It does this by providing incentives, such as grants for certain new animal drugs for MUMS use to help with costs associated with drug testing (FDA, 2013; Health Canada-1, 2009; FDA/HHS, 2007). For example, it allows companies sponsoring drugs for approval to receive tax credits (50% of the clinical testing expenses), or owners of animals used for clinical testing to apply for a tax break (FDA, 2013; MUADP-1, 2013; FDA/HHS, 2007). While this is U.S. legislation, more MUMS drugs are expected to become available and known to Canadian veterinarians, but not necessarily registered in Canada since the same incentives (to register drugs) do not currently apply (Health Canada-1, 2009).

## **2.2 Veterinary Drugs Directorate**

Health Canada is responsible for protecting human and animal health and for the safety of all foods sold in Canada (Health Canada-3, 2013; Agri-Food Canada, 2010; Health Canada, 2006). Through the Veterinary Drugs Directorate (VDD), Health Canada monitors and evaluates the safety, quality, and effectiveness of veterinary drugs, sets guidelines, and promotes practical use of veterinary drugs administered to animals (Grignon-Boutet et al., 2008; Health Canada, 2006). The VDD has a key role in ensuring our food, such as milk, meat, eggs, fish, and honey, are safe from animals treated with veterinary drugs (Health Canada, 2006; Health Canada-2, 2004). The VDD works in partnership with other federal agencies, provincial governments, industry, producers, and farmers (Agri-Food Canada, 2010; Health Canada-2, 2004). They also ensure that veterinary drugs sold and used in Canada are safe and effective for animals being treated (Grignon-Boutet et al., 2008; Health Canada, 2006).

The VDD is comprised of a multi-disciplinary team of people including those in veterinary medicine, biology, parasitology, aquaculture, pharmacology, toxicology, environmental science, as well as policy, finance, and administration (Health Canada, 2006). Each division specializes on a specific task or role in the organization. The VDD works at a national and international level in collaboration with drug manufacturers, veterinarians, and livestock producers (Agri-Food Canada, 2010; Health Canada-2, 2004) to work towards harmonization of the technical requirements for veterinary drug approvals (i.e. with the United

States FDA/CVM), and to increase the availability of safe veterinary drugs for food animals in Canada (Agri-Food Canada, 2010). The organization focuses on 3 key aspects: (1) development of a prioritized list of approved drugs with the United States' Maximum Residue Limits (MRLs) requiring Canadian MRLs; (2) provide information and guidance for industry; and (3) enhance policies, guidelines, and regulatory requirements (Agri-Food Canada, 2010).

### **2.2.1 Drug Approval Process: Maximum Residue Limits and Withdrawal Periods**

Any drug desired for use in Canada, whether permitted here or in another country, has to be acknowledged and reviewed by the VDD. Before any drug is approved for use in Canada, Health Canada, through the VDD, evaluates it for product quality, efficacy, and animal and human safety, including toxicity, metabolism, residue, and microbiology information (Health Canada-3, 2013; Health Canada, 2011; Health Canada-1, 2009; Health Canada, 2002). Health Canada also determines the conditions of sale and label requirements (Health Canada-1, 2009; Health Canada-2, 2009; Health Canada, 2002). Each drug must fully satisfy requirements under the *Food and Drugs Act and Regulations* (Health Canada-3, 2013; Health Canada, 2011; Health Canada-2, 2009; Health Canada, 2006). Decisions are made based on scientific data, with the safety of people, animals, and the environment always in mind (Health Canada-2, 2009; Health Canada-2, 2004). A new veterinary drug is approved for sale in Canada only if Health Canada is satisfied the manufacturer has supplied evidence that: the drug is safe for the animals being treated; is effective for treating the target disease or condition; does not leave potentially harmful residues that could pose any health hazard to humans eating food products from treated animals; the drug is manufactured according to strict specifications and remains stable up to its expiry date; and there is a suitable regulatory method to monitor its use (Health Canada, 2011; Health Canada-1, 2004).

The *Food and Drugs Act and Regulations* list other recommendations designed to address food safety and advise the public of potential health risks due to drug residues in food. They prohibit the sale of meat for human consumption if it contains specified or unapproved drugs (Grignon-Boutet et al., 2008). Each approved drug product (animal or human) is issued a DIN specific to that drug (Health Canada, 2011; Health Canada-2, 2009). This DIN indicates the product has passed a review of safety, efficacy, and quality, and shows that Health Canada has

approved the formulation, labeling, and instructions for use (Health Canada-2, 2009). Any drug product sold in Canada without a DIN is in violation of Canadian law (Health Canada-2, 2009). In special circumstances the VDD may authorize the sale of limited quantity of an unlicensed drug to a veterinarian for use in a specific emergency (Health Canada, 2011; Health Canada-1 2009; Health Canada, 2006). During the safety evaluation and approval process of drugs used in food-animals, maximum residue limits and withdrawal periods are established.

A **maximum residue limit** (MRL), also known as tolerance level in the United States, is the maximum amount of residue that is legally acceptable or allowed to remain in the tissues or food products of animals treated with veterinary drugs (Health Canada-1, 2013; Health Canada-3, 2013; Merck Inc, 2011; Health Canada-2, 2009; Health Canada-1, 2004). It is the concentration that is considered safe for humans to consume, based on food consumption factors, resulting in total residue ingestion lower than the acceptable daily intake, and should pose no adverse health risks if ingested daily over a lifetime (Health Canada-1, 2013; Health Canada-3, 2013; Merck Inc, 2011; Health Canada-1, 2004). MRLs are established after the VDD has extensively reviewed scientific data and determined that food containing these veterinary drug residues up to the allowable levels are safe for human consumption (Health Canada-1, 2013; Health Canada-3, 2013; Health Canada-2, 2009; Health Canada-1, 2004). MRLs are set in each animal species and in each tissue the drug is approved for use in (Merck Inc, 2011). In cases where no MRL has been established for specific veterinary drugs (unapproved drugs, or drugs used extra-label), no detectable residues are permitted in animal-derived food products (Health Canada-1, 2013; Health Canada-2, 2009). Food from animals treated with unapproved drugs could contain potentially harmful residues for those eating the food (Health Canada-1, 2004). Health Canada is actively working to have MRLs established for drug products that could be used for food-producing animals (Health Canada-1, 2013; Health Canada-2, 2009).

The **withdrawal period** is the interval between the time of last drug administration (of the label or recommended dose) and the time when the animals can safely be slaughtered for human consumption, or when food products from the animals can be used for consumption (Health Canada, 2011; Merck Inc, 2011; Cattet, 2003). The withdrawal period is determined on the basis of an existing MRL. This time allows the drug and its residues to deplete to levels that do not pose health risks to humans eating the food (Health Canada, 2011; Merck Inc, 2011; Craigmill et al., 2004). Animals slaughtered within this time period may be unsafe for human

consumption, whereas animals killed after the withdrawal period should have no adverse risk to humans (Cattet, 2003).

MRLs and withdrawal periods may be different in other countries because procedures for establishing such factors are not the same internationally (Health Canada-1, 2013; Craigmill et al., 2004). The drug approval process, animal husbandry practice, and legislation in Canada may vary from that of other countries (including the U.S.) (Health Canada-1, 2013). Food consumption patterns may vary according to culture and there may be differences in methods used to measure residues or in the way MRLs are calculated (Health Canada-1, 2013; Health Canada-1, 2004). Health Canada is actively working to standardize MRLs, but sometimes conclusions are made that result in setting different MRLs for veterinary drugs (Health Canada-1, 2013). In Canada, the VDD bases veterinary drug marketing, labeling, and MRLs on scientific evaluation that includes views from other jurisdictions (Health Canada-1, 2013).

The Canadian Food Inspection Agency (CFIA) is responsible for enforcing the food safety policies and standards set by Health Canada (Health Canada-1, 2004). In the case of monitoring for drug residues, tissue samples are often tested from food processing plants or from the shelf in a grocery store. Food inspection agencies, such as the CFIA, regularly monitor for drug residue violations (Health Canada, 2011; Health Canada-2, 2009). A **violative** or **non-compliant drug residue** is the presence of a drug in a food product that is higher than the set MRL for that specific veterinary drug (Health Canada-1 2009; Health Canada-2, 2009). When a potential health risk is found in marketed foods, Health Canada, through the VDD, assesses this risk to Canadians (Health Canada-3, 2013; Health Canada-2, 2009; Health Canada-2, 2004; Health Canada, 2002). The CFIA is permitted, under the *Food and Drugs Act* to take corrective action, if necessary (Health Canada-3, 2013), such as seizure of goods, food recalls, or even closure of facilities, depending on the circumstances.

### 2.2.2 Extra-Label Drug Use

As stated earlier, veterinarians use unapproved drugs in minor species. This is referred to as extra-label drug use, or off-label use. **Extra-label drug use (ELDU)** is the use of a drug in a way that is not in accordance from that described on the approved drug label (Merck Inc, 2011; Health Canada-1, 2009; Health Canada-2, 2009; MUADP, 2009; Grignon-Boutet et al., 2008;

Cattet, 2003). This includes using an approved drug (contains a DIN) in a species that is not included on the drug label, used at a different dose, administered in a different way, used for longer or shorter period of time, used in a different age group, and/or the use of the drug for diseases that are not included in the drug label (Merck Inc, 2011; Health Canada-2, 2009; Craigmill et al., 2004). For example, tulathromycin, a drug approved for treating respiratory infection in cattle, can be used in a minor species, such as bison or deer. Some drugs, including tulathromycin, can be used off-label in food-producing animals, providing certain conditions are met; however, Health Canada prohibits off-label use of a small number of veterinary drugs, specifically those at high risk for developing antimicrobial resistance or other risks to public health (Health Canada-2, 2009; Grignon-Boutet et al., 2008).

ELDU typically occurs when there is a lack of safe or effective approved drugs (or dosages) to treat a specific species or condition (Health Canada-1, 2009; Health Canada-2, 2009; Health Canada, 2002) or for economic and other welfare considerations (Health Canada-2, 2009). ELDU is more common in minor species because there are so few approved drugs for minor species use (Health Canada-1, 2009; Health Canada-2, 2009). In a survey conducted in 1990 (plans to conduct a following survey in 2003 were suspended due to cost constraints), ELDU of veterinary drugs typically involved the administration of drugs (mostly antibiotics) at higher doses and/or in species not included on the drug label (Health Canada-1, 2009). While these results may no longer be current, it does provide readers with an indication of the scope of ELDU in minor species.

Although the VDD encourages veterinarians to use approved drugs following label recommendations whenever possible (Health Canada, 2011; Health Canada-1, 2009), they acknowledge that ELDU is important in veterinary medicine (Health Canada-1, 2009; Health Canada-2, 2009). ELDU is necessary for animal welfare and for the proper and humane care of sick animals (Health Canada-1, 2009). Even so, this practice raises concerns. Two primary public health concerns and food safety risks exist that relate to ELDU: (1) the possibility of leaving violative and potential harmful drug residues in food products (or the environment); and (2) the potential for development and spread of drug-resistant pathogens of bacteria, otherwise referred to as antimicrobial resistance (Health Canada-1, 2009; Health Canada-2, 2009; Wang, 2009; Grignon-Boutet et al., 2008).



### **a. Extra-Label Drug Use and Drug Residues**

Veterinary **drug residues** are the small amounts of veterinary drugs that may remain in animal products after a drug has been used in an animal (Merk Inc, 2011; Craigmill et al., 2004). This includes any degradation products or metabolites of the drug. Drug residues are a major concern when it comes to using drugs in food-animals (Cattet, 2003), and are even more of a concern when using drugs extra-label. Each time a veterinary drug is used in a food animal, there is potential to leave drug residues in the tissues that we consume (Health Canada-1, 2009). The administered drug distributes throughout the body, to the target area (if treating a lung infection, it will go to the lung), but it will also distribute to other areas, such as muscle –which is what people generally eat, before the drug is eliminated.

When a drug is used off-label, the edible tissues of that particular animal species may be unfit for human consumption because the necessary safety evaluations have not been done in that particular animal species (Merck Inc, 2011; Cattet, 2003). This is a concern for minor species, where drugs are being used that have not been approved. MRLs and withdrawal periods may no longer be applicable, and could potentially cause risks to consumers (Cattet, 2003). Disregarding withdrawal times or incorrect use of a veterinary drug can lead to unwanted residues in food products, potentially causing adverse effects to consumers, such as allergic reactions (Wang, 2009). There is a significant level of uncertainty regarding the presence (or absence) of drug residues; at what concentration, or what effect they have in a person if ingested. By extrapolating data obtained from other species to estimate drug behaviour, the chance of violating MRLs in tissues for human consumption is high (Cattet, 2003).

### **b. Extra-Label Drug Use and Antimicrobial Resistance**

**Antimicrobial resistance (AMR)** occurs when an antimicrobial is no longer effective in killing or inhibiting the growth of a targeted microorganism (Health Canada-1, 2004; Health Canada, 2003). AMR is an emerging global health issue for both animals and humans (CVMA, 2009; Health Canada, 2003; Health Canada, 2002). Resistance occurs when antibiotics are widely used and where bacteria can readily be passed between individuals (Health Canada, 2003). The longer an antimicrobial is used, the more likely resistance will develop (Health Canada-1, 2009; Health Canada, 2003). The issue with AMR is that it potentially threatens our ability to fight

infections in animals and humans (Health Canada-1, 2009; Health Canada-1, 2004; Health Canada, 2003). This is an important problem because effective antibiotics for treating infections could become fewer, making treatment more challenging (Health Canada-1, 2009; Health Canada-1, 2004; Health Canada, 2003), and may lead to use of more expensive drugs (Health Canada, 2003; Health Canada, 2002). This is also important because bacteria sometimes spread from animals to humans. Some of these bacteria cause illness in humans or transfer their resistant genes to human bacteria (Health Canada-1, 2009; Health Canada, 2003; Health Canada, 2002).

Over-use and/or inappropriate use of antimicrobials can result in increased antimicrobial resistance (Health Canada-1, 2004; Health Canada, 2003). Over-the-counter availability of veterinary antimicrobials may add to the risks associated with antimicrobial use (Health Canada, 2002). Inappropriate treatment includes use for the wrong disease, wrong dose, for incorrect periods of time, or route of administration (Health Canada, 2002). Minimizing use of antimicrobial agents is a vital solution to control and overcome AMR (Health Canada-1, 2009). The VDD actively assesses ways to regulate antimicrobial use and is considering the inclusions of risk for resistance as part of the regulatory review process for antimicrobials, as well as developing a surveillance system for antimicrobial use and resistance (Health Canada, 2003; Health Canada, 2002).

Due to the concern for the development of resistance, ELDU of some specific antimicrobial agents is restricted or prohibited (Merck Inc, 2011; Health Canada-1, 2009; Grignon-Boutet et al., 2008). Veterinarians should prescribe antimicrobials cautiously, especially for ELDU (CVMA, 2009; Health Canada, 2002). If an antimicrobial is used extra-label, the veterinarian must provide the appropriate dose, route, frequency, duration, and withdrawal time to avoid any risk to humans (CVMA, 2009). It is a matter of balancing between “maximizing animal health and welfare, minimizing bacterial resistance, and conserving antimicrobial efficacy” (CVMA, 2009). Antimicrobials should not be used extra-label unless there is support for efficacy, dosage regimen, indication, and withdrawal times (CVMA, 2009). Withdrawal times can be estimated in consultation with the Canadian Global Food Animal Residue Avoidance Database (CgFARAD) (CgFARAD, 2013; CVMA, 2009).

### **2.2.3 Food Animal Residue Avoidance Databank (FARAD)/Canadian Global FARAD**

The Food Animal Residue Avoidance Databank (FARAD) provides information related to approved animal drugs, including tissue residues and withdrawal time estimates for those drugs used off-label in food animals (FARAD, 2013; Health Canada-1, 2009; Craigmill et al., 2004). FARAD is a computer database designed to provide livestock producers, veterinarians, and other specialists with practical information on how to avoid residue problems (FARAD, 2013; Craigmill et al., 2004). They provide accurate information regarding drug disposition in animals prior to slaughter. Member countries, Canada, the United States, Australia, and Europe, have web-based access to the FARAD database and share all relevant drug and chemical information and tolerance data (FARAD, 2013; Health Canada-1, 2009). In general, the program provides effective residue avoidance and quality assurance of products used in animals, such as drugs, pesticides, and environmental contaminants (FARAD, 2013). FARAD maintains up-to-date drug label information and MRLs on all drugs and pesticides used in tissues, eggs, and milk (FARAD, 2013). Where a MRL is unknown (drug used off-label), all possible data is used for estimating a withdrawal time, including information on pharmacokinetics. Whenever a decision is made, withdrawal time estimates are always increased in order to help ensure the residues will be below some acceptable regulatory limit by the end of the recommended withdrawal period (Craigmill et al, 2004; Cattet, 2003).

Canada is part of a global FARAD program (CgFARAD), which provides data (ex: pharmacokinetic, residues) on drugs and chemicals unique to Canada's environment and production practices, and is a source of local regulatory expertise in regards to import and export food safety (CgFARAD, 2013; FARAD, 2013; Health Canada-1, 2009). It is based out of the Western College of Veterinary Medicine (WCVM) in Saskatoon SK, and the Ontario Veterinary College (OVC) in Guelph, ON (CgFARAD, 2013; Health Canada-1, 2009). The purpose of the CgFARAD is not to promote extra-label drug use, but to protect the food supply when it is necessary for veterinarians to use drugs extra-label (Health Canada-1, 2009). Using information from CgFARAD does not exonerate the user from responsibility.

## 2.3 Tulathromycin

Tulathromycin is a semi-synthetic macrolide antibiotic approved for use in treating bovine and swine bacterial respiratory disease (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). More specifically, tulathromycin is used for bovine respiratory disease (BRD) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* (*Haemophilus somnus*) and *Mycoplasma bovis* in cattle, and swine respiratory disease (SRD) associated with *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* in pigs (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Pfizer Inc, 2005; EMEA, 2003). It is marketed by Pfizer under the trade name *Draxxin* (APVMA, 2007; Evans, 2005; Pfizer Inc, 2005; EMEA, 2003), which is formulated as a slow release, single injectable dose therapy, containing 100 mg of tulathromycin per mL (Drugs.com, 2013; Evans, 2005; FDA/CVM, 2004). In addition to being used as an effective treatment option for respiratory disease in cattle and pigs, tulathromycin is used as a preventative measure in cattle feedlots to reduce risk of contracting respiratory disease (Drugs.com, 2013; Evans, 2005; EMEA, 2003). *Draxxin* is approved for use in bovine and swine in 25 countries, including Canada, the United States, Europe, and Australia (Drugs.com, 2013; APVMA, 2007; Pfizer Inc, 2005; EMEA, 2003).

### 2.3.1 Chemistry

The chemical structure of tulathromycin (**Figure 2.1**) contains three polar amine groups, distinguishing it from other macrolides, including azalides and ketolides, and thus designating it a member of the newly assigned triamilide subclass of macrolide antibiotics (APVMA, 2007; Evans, 2005; FDA/CVM, 2004; EMEA, 2003). Tulathromycin was developed solely for veterinary use (Evans, 2005; EMEA, 2004; EMEA, 2003). Some macrolides require multiple injections in order to reach the desired therapeutic effect (Evans, 2005; Gáler et al., 2004). However, *Draxxin*, the commercially available form of tulathromycin, is designed as a long acting formulation. Partially due to its unique chemical structure, tulathromycin reaches greater penetration into the targeted bacteria and is able to remain in the target tissue (lung) for longer periods of time after a single administration (Evans, 2005; Gáler et al., 2004). Tulathromycin is lipophilic, basic (pKa values of 8.5, 9.3, and 9.8), and has a low degree of ionization, which

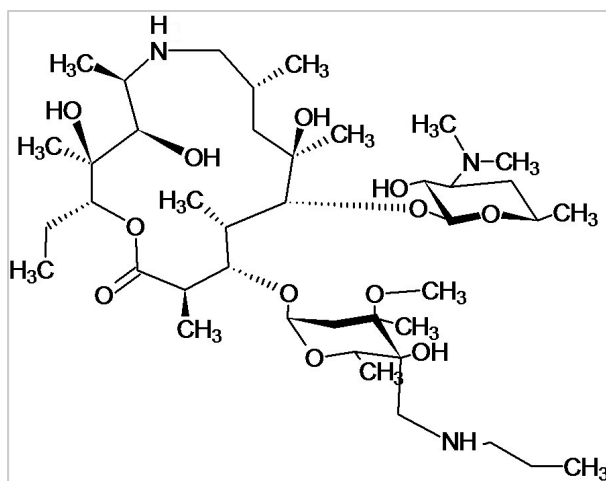
allows for extensive tissue penetration (Reeves, 2012; Benchaoui et al., 2004; Nowakowski et al., 2004; Zhanel et al., 2001). Tulathromycin accumulates in immune cells (phagocytes), which travel to the lung tissue in response to inflammation, and is slowly released into the extracellular environment where it is available to attack respiratory pathogens (Evans, 2005; Kilgore et al., 2005; Zhanel et al., 2001).

In solution, tulathromycin equilibrates into a stable mixture of two isomers, CP-472,295 (15-ring member) and CP-547,272 (13-ring member) in a 9:1 ratio, respectively (Drugs.com, 2013; APVMA, 2007; EMEA, 2004; EMEA, 2003). The IUPAC chemical names of each isomer are: 2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-13-[[2,6-dieoxy-3-C-methyl-3-O-methyl-4-C-[(propylamino)methyl]- $\alpha$ -L-ribo-hexopyranosyl]oxy]-2-ethyl-3,4,10-trihydroxy-3,5,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)- $\beta$ -D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one (CAS 217500-96-4) (A; CP-472,295)

and

2R,3R,4R,5R,8R,10S,11S,12R)-11-[[2,6-dieoxy-3-C-methyl-3-O-methyl-4-C-[(propylamino)methyl]- $\alpha$ -L-ribo-hexopyranosyl]oxy]-2-[(1R,2R)-1,2-dihydroxyl-1-methylbutyl]-8-hydroxy-3,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)- $\beta$ -D-xylo-hexopyranosyl]oxy]-1-oxa-4-azacyclotridecan-13-one (CAS 280755-12-6) (B; CP-547,272) (Drugs. com, 2013; APVMA, 2007; EMEA, 2004).

The two isomers are considered the pharmacologically active form of the drug (Boner et al., 2011; Clotheir et al., 2011; APVMA, 2007; EMEA, 2004) and also referred to as CP-472,295(e) (APVMA, 2007; EMEA, 2003).



**Figure 2.1:** Tulathromycin A ( $C_{41}H_{79}N_3O_{12}$ ; 806.23 g/mol).

### 2.3.2 Mechanism of Action

Like other macrolides, tulathromycin acts by inhibiting protein synthesis of bacteria (APVMA, 2007; Evans, 2005; Pfizer Inc, 2005; FDA/CVM, 2004). It binds to bacterial 50S ribosomal subunits stimulating the dissociation of peptidyl-tRNA from the ribosome during the translocation process (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Pfizer Inc, 2005; EMEA, 2004; FDA/CVM, 2004; EMEA, 2003), which leads to a disruption of bacterial protein synthesis.

Tulathromycin is primarily bacteriostatic, but may be bactericidal at higher concentrations against some pathogens (Drugs.com, 2013; Pfizer Inc, 2005). It is a broad-spectrum antibiotic, with *in vitro* activity against certain gram-negative and gram-positive bacterial pathogens, including the bacterial pathogens associated with bovine and swine respiratory disease, such as *Pasteurella*, *Mannheimia*, *Actinobacillus*, and *Mycoplasma* species (APVMA, 2007; Pfizer Inc, 2005; FDA/CVM, 2004).

### 2.3.3 Pharmacokinetics in Bovine and Swine

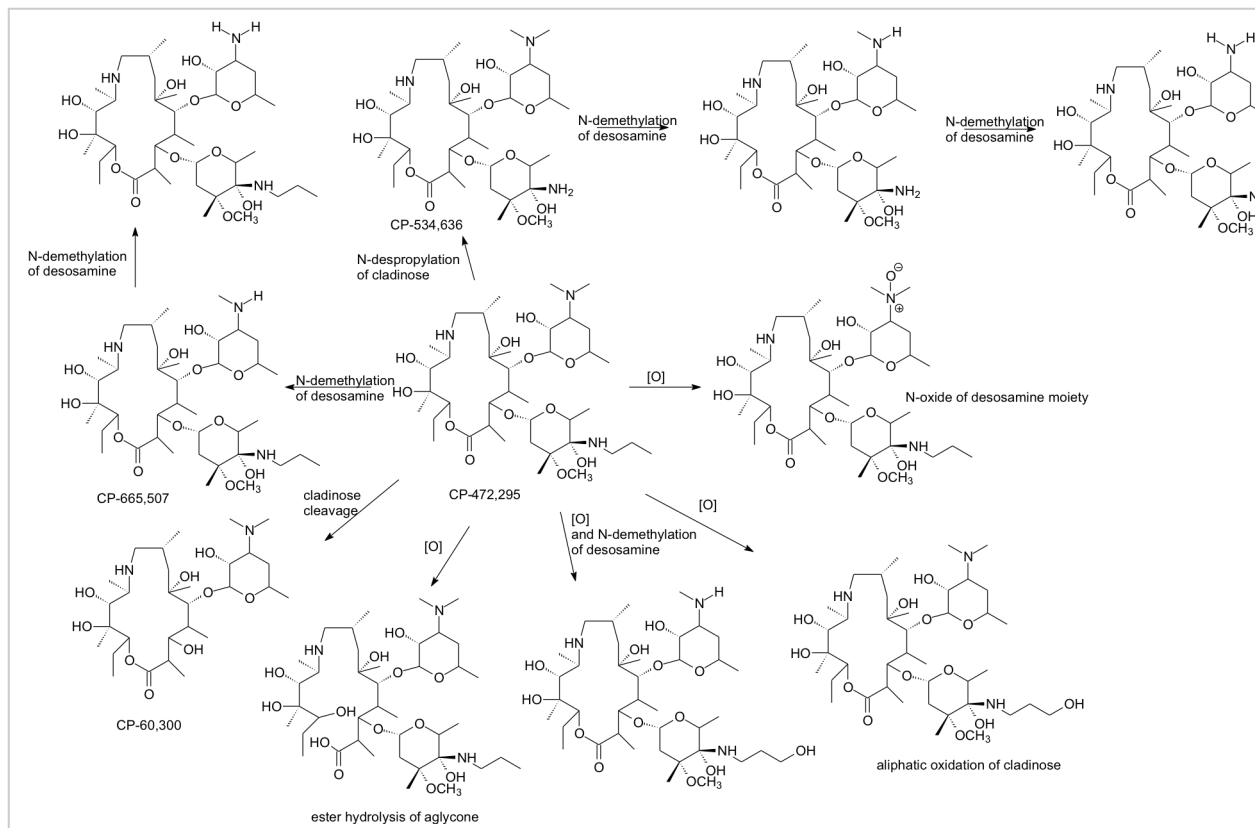
The pharmacokinetic profile of tulathromycin has been characterized extensively in bovine (Evans, 2005; Gáler et al., 2004; Nowakowski et al., 2004) and swine (Huang et al., 2012; Wang et al, 2012; Benchaoui et al., 2004; Gáler et al., 2004). More recently, tulathromycin has been studied in goats (Romanet et al, 2012; Clothier et al., 2011; Young et al., 2010) and foals

(Scheuch et al., 2007; Venner et al., 2007). These studies mainly focus on the pharmacokinetics and characterization of tulathromycin after a single injection (Young et al., 2010; Scheuch et al., 2007; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004). In these species, tulathromycin is rapidly absorbed and rapidly and widely distributed to tissues with accumulation occurring in the lungs (Young et al., 2010; Scheuch et al., 2007; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004). Elimination is slow (but relatively complete) (Scheuch et al., 2007; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004), which provides prolonged drug concentrations in the lungs and, thus, continued activity against the targeted bacteria (APVMA, 2007; EMEA, 2004).

More specifically, in studies using cattle and swine, peak plasma concentrations ( $C_{max}$ ) are reached within 1 hour after injection (Huang et al., 2012; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004) following a single 2.5 mg/kg bw subcutaneous injection in cattle, and intramuscular injection in pigs. Bioavailability is >90% in cattle and >80% in pigs (Drugs.com, 2013; Evans, 2005; Pfizer Inc, 2005; EMEA, 2004; EMEA, 2003). Lung tissues accumulate tulathromycin at higher concentrations than plasma (Drugs.com, 2013; Scheuch et al., 2007; Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004), affording effective single dose treatment for respiratory infections of cattle and pigs (APVMA, 2007; Evans, 2005). In both species, tulathromycin distributes extensively to body tissues, as demonstrated by a relatively large apparent volume of distribution (>10 L/kg) and a long plasma elimination half-life (3-5 days in cattle, and 2-3 days in pigs) (Drugs.com, 2013; Evans, 2005; Pfizer Inc, 2005; EMEA, 2004; Gáler et al., 2004; EMEA, 2003). Tulathromycin is eliminated primarily as unchanged parent compound by biliary excretion (Drugs.com, 2013; APMVA, 2007; Evans, 2005; Nowakowski et al., 2004).

Metabolism of tulathromycin is minimal, with the majority of the drug (>90%) being excreted as unchanged parent drug (APVMA, 2007; Evans, 2005; EMEA, 2004; FDA/CVM, 2004; Nowakowski et al., 2004). The parent isomers contribute to most of the residues in edible tissues, and in the urine and feces (APVMA, 2007; Evans, 2005; Nowakowski et al., 2004). Even though metabolism is minimal, the metabolites of tulathromycin have been identified and are shown in **Figure 2.2**. There are 10 known metabolites of tulathromycin, along with the 2 parent isomers, making the total residue count at 12 (APVMA, 2007). Metabolism involves the following processes: N-demethylation or N-oxidation (of the desosamine portion of the

molecule); cleavage of the modified cladinose moiety; N-depropylation (of cladinose); ester hydrolysis (of the macrocyclic ring); and other combinations of oxidation or demethylation (APVMA, 2007; EMEA, 2004). Although parent drug is the predominant residue, a radioactive residue study in cattle indicated the major metabolite was the N-depropylation of the cladinose, which is excreted into the bile and made up approximately 16% of total radioactive residue (APVMA, 2007; EMEA, 2004; EMEA, 2003). In pigs, N-oxidation of desosamine in the skin/fat was the major metabolite, making up ~20% of total radioactive residue (APVMA, 2007; EMEA, 2003). All other tissues and excreta analyzed (urine, feces, bile, liver, kidney, muscle, fat, skin/fat, and injection site residues) contained parent drug and tulathromycin metabolites at less than 10% of total radioactive residue (APVMA, 2007). The major component in the liver and bile of each species (also in all other edible tissues of the target species) was the unchanged drug (Evans, 2005; EMEA, 2004).



**Figure 2.2:** Metabolic pathway of tulathromycin in cattle and swine. Adapted from: Australian Pesticides and Veterinary Medicines Authority. Public Release Summary: Draxxin injectable solution, APVMA Product Number 59304, *p16* (APVMA, 2007).



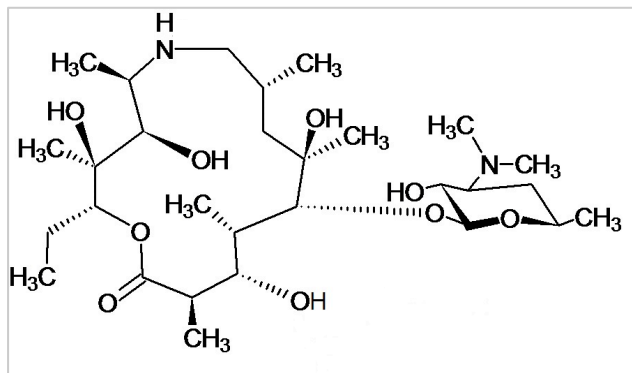
### 2.3.4 Maximum Residue Limits and Withdrawal Periods in Bovine and Swine

An important metabolite, formed by the cleavage of the cladinose, results in a common hydrolytic fragment, CP-60,300 (**Figure 2.3**). The FDA and Health Canada recognize this metabolite, expressed as tulathromycin equivalents, as the marker residue in edible tissues in bovine and swine (Health Canada-2, 2013; EMEA, 2004). A **marker residue (MR)** is a residue, either the parent drug or a metabolite (or some combination), that is selected to monitor the total residue concentration (Merck Inc, 2011; Craigmill et al., 2004). The two tulathromycin isoforms can be converted to the CP-60,300 common fragment by acid hydrolysis (Romanet et al., 2012; Boner et al., 2011; Clotheir et al., 2011; APVMA, 2007; EMEA, 2004).

Using the established MR and other residue and safety assessments of tulathromycin, maximum residue limits (MRLs) and withdrawal periods are established, as stated above (**Section 2.2.1**). In Canada, the MRL for tulathromycin in cattle are as follows: 4.0 ppm (4000 µg/kg) kidney, 2.0 ppm liver, 1.0 ppm muscle tissue; and in pigs: 5.0 ppm kidney, 4.0 ppm liver, 1.5 ppm muscle (Health Canada-2, 2013). In the United States, the MRL in cattle liver is 5.5 ppm and 15.0 ppm in swine kidney (Pfizer Inc, 2005). Europe and Australia have similar MRLs for both bovine and swine, which are: 0.1 ppm fat; 3.0 ppm liver; 3.0 ppm kidney tissue (APVMA, 2007; EMEA, 2004; EMEA, 2003).

The withdrawal period reflects the time needed for the MR to deplete below the established MRL in the slowest-depleting (99<sup>th</sup> percentile) edible tissues of animals (Craigmill et al., 2004). Liver tissue in cattle and kidney tissue in pigs had the highest concentration and the longest depletion profile of tulathromycin (APVMA, 2007; Pfizer Inc, 2005). These tissues will determine the withdrawal period for tulathromycin in these species and will determine how long the animals need to be kept before slaughter for human consumption (APVMA, 2007). The product labeling for *Draxxin* in Canada lists a withholding period of 44 days in cattle and 8 days in swine (Drugs.com, 2013). In other words, tulathromycin should not be used in animals less than 44 days and 8 days before slaughter for human consumption in cattle and pigs, respectively. When using these recommended withholding periods, tulathromycin residues in cattle and pigs are covered by the MRLs and are considered safe for consumption (APVMA, 2007). A withdrawal period of 18 days in cattle and 5 days in swine is assigned for tulathromycin use in the United States (Pfizer Inc, 2011) and in Australia it is 35 days in cattle and 14 days when used

in pigs (APVMA, 2007). The withdrawal period for tulathromycin in Europe is 49 days for cattle and 33 days for pigs (EMA, 2003).



**Figure 2.3:** Common fragment and marker residue of tulathromycin, CP-60,300.

## 2.4 Tulathromycin Analysis

### 2.4.1 Mass Spectrometry

The assessment of tulathromycin pharmacokinetics and tissue withdrawal in bison and deer requires the development of a sensitive and accurate analytical method. Microbiological and immunological assays are conventional macrolide screening methods, but lack adequate specificity and precision for regulatory purposes (Chico et al., 2008; Berrada et al., 2007; Horie et al., 2003; Codony et al., 2002; Draisci et al., 2001). Chromatographic methods, combining liquid chromatography (LC) with ultra violet (UV), fluorescence, or electrochemical detectors, are appropriate alternatives, but are not as accurate for the determination and confirmation of macrolide residues (Wang, 2009; Berrada et al., 2007; Codony et al., 2002). Since the last decade, liquid chromatography coupled with mass spectrometry (LC-MS) has become an essential technique in residue and food analysis (Mohamed et al., 2011; Wang, 2009; Chico et al., 2008; McGlinchey et al., 2008) and in pharmacokinetic assessments (Mulvana, 2010; Berna et al., 2004). LC-MS is an analytical technique that combines the separation power of high-performance liquid chromatography (HPLC) with the sensitivity and specificity of the mass spectrometer (Mohamed et al., 2011). This analytical method is sensitive and specific for accurately measuring the marker residue at concentrations at or below the maximum residue limit

(Mohamed et al., 2011; Wang, 2009; APVMA, 2007). Mass spectrometry analysis provides important information about the analytes (substance or chemical component undergoing analysis) including structure, purity, and composition (Mohamed et al., 2011; Wang, 2009; Chico et al., 2008; McGlinchey et al., 2008; Berrada et al., 2007). LC-MS methods have been reported for tulathromycin in plasma and tissue samples in cattle, swine, and other species (Huang et al., 2012; Boner et al., 2011; Young et al., 2010; Scheuch et al., 2007; Benchaoui et al., 2004; Gáler et al., 2004; Nowakowski et al., 2004).

For tulathromycin, methods have been used to measure parent tulathromycin (CP-472,295) residues in tissues using HPLC-MS (Huang et al., 2012; APVMA, 2007; Scheuch et al., 2007; Gáler et al., 2004). A second analytical method measures the common fragment, CP-60,300, a metabolite of the parent compound (Romanet et al., 2012; Boner et al., 2011; Clothier et al., 2011; APVMA, 2007; EMEA, 2003). As stated above (**Section 2.3.4**), the common hydrolytic fragment of tulathromycin has been established as the marker residue in edible tissues for monitoring and surveillance purposes. This residue is defined as the sum of tulathromycin and its metabolites that are converted by acid hydrolysis to CP-60,300, expressed as tulathromycin equivalents (Boner et al., 2011; Clothier et al., 2011; APVMA, 2007; EMEA, 2004). Some reports found analyzing the hydrolytic fragment yielded similar results to those observed by the parent compound procedure (APVMA, 2007; EMEA, 2004). Others state that the acid digest fragment analytical method accounts for a higher percentage of total residues than the method for measuring parent tulathromycin (Boner et al., 2011; EMEA, 2004), yet since the residue decline profile of CP-472,295 and CP-60,300 parallels the depletion curves of total residues in most edible tissues, both analytes are acceptable as the residue definition of tulathromycin (APVMA, 2007).

#### **2.4.2 Solid Phase Extraction**

Sample preparation prior to LC-MS analysis, including extraction, clean-up, and concentration, are very important in the overall analysis and quantification of macrolides (Wang, 2009; McGlinchey et al., 2008). Matrices contain many possible substances that can interfere with the analytical method and require their removal prior to analysis (McGlinchey et al., 2008). For example, liver and muscle are complex matrices because of their high fat and protein content,

which may interfere with the identification of the selected analytes (McGlinchey et al., 2008). The sample preparation portion of the analysis is often the most critical and difficult part, both in terms of time involved and the difficulty of extracting the desired analyte from the matrix. In addition, each matrix has its own unique challenges. In most cases, macrolides, including tulathromycin are extracted from samples by removal of proteins and fat using organic solvents (liquid-liquid extraction), and then further cleaned-up and pre-concentrated using solid-phase extraction (SPE) (Berrada et al., 2007; Horie et al., 2003; Codony et al., 2002; Draisci et al., 2001). SPE involves passing a liquid extract through a solid sorbent cartridge, which selectively retains the analyte of interest on the cartridge (Wang, 2009; McGlinchey et al., 2008). The cartridge is washed to remove matrix components, and the macrolides are then eluted with an organic solvent, commonly acetonitrile or methanol, and concentrated down by evaporation to an appropriate volume for LC-MS analysis (Wang, 2009). SPE is beneficial because it allows greater clean-up of samples and reduces matrix effects and eliminates interfering substances, which may occur between the sample and analyte of interest (Wang, 2009). This process also concentrates the analytes so as to achieve sensitivity with low detection limits (Wang 2009).

## **2.5 Purpose of the Research**

Respiratory illnesses are a problem in deer (Dyer et al, 2004) and are increasingly becoming a leading cause of death in bison (Janardhan et al., 2010; Dyer et al., 2008). Although tulathromycin has been used off-label in these species, a review of the literature failed to find reference to a study of this antibiotic in these animals. Pharmacokinetics and PK research provide a means to design optimal dosage regimens and assists in our understanding of drug kinetics and mechanisms of drug effects (Toutain et al., 2010). Thus, following PK investigation in bison and white-tailed deer, we will generate information that may help improve appropriate dose size selection and frequency of administration of tulathromycin, and establish a more effective therapeutic treatment for these species. Further, analysis of tissue residues will determine drug distribution and how long the drug persists, which will provide information regarding withdrawal times of tulathromycin in these species, which is important for consumer safety. Significant interspecies differences in PK processes and tissue distribution warrant specific investigations in the species of interest.

### **2.5.1 Objectives**

The two primary objectives for the thesis research are: (1) To conduct a pharmacokinetic study to investigate proper dosage regimens for tulathromycin in bison and white-tailed deer; and (2) To conduct a tissue depletion study to provide appropriate withdrawal time information for tulathromycin use in bison and white-tailed deer. In order to complete the above objectives, an analytical method is first needed to quantify tulathromycin in serum and in tissues. Outcomes from these studies will provide important information to veterinarians, to producers, and as a food-safety issue to consumers of these animals. Information about tulathromycin is important to bison and deer producers who may incur significant losses each year due to respiratory disease. In approved species, tulathromycin is an effective treatment of respiratory disease, but clinical evidence also suggests tulathromycin as a chemopreventative against respiratory disease (Drugs.com, 2013; Evans, 2005; EMEA, 2003).

### 3. METHOD DEVELOPMENT

Liquid chromatography-mass spectrometry (LC-MS) was chosen as the analytical method for tulathromycin analysis in deer and bison serum and tissues due to its sensitivity and specificity. As with many analytical undertakings, method development for tulathromycin quantification in bison and deer serum and tissues came with some challenges. Much of the laboratory research was done at the Canadian Food Inspection Agency's Centre for Veterinary Drug Residues (CFIA/CVDR), Saskatoon laboratory. A method was developed and validated in bison serum first, followed by deer serum. Application of the method to deer serum and tissues required some adjustments to the extraction and analysis procedures relative to the bison. Differences noted between bison and deer may relate to species differences in serum proteins and dietary differences; however, no information was found in the published literature regarding similar problems. The tissue method that was developed and validated in deer lung and muscle also appeared to work well in other tissues, including bison lung tissue.

#### 3.1 Bison Serum Extraction Method

A solid-phase extraction method was selected as a starting point for tulathromycin extraction in bison (and deer) serum. The method, adopted from Scheuch et al. (2007) and Gálér et al., (2004), consisted of using 0.5 mL of serum diluted with 0.5 mL of water (pH 7.0). The solid-phase extraction (SPE) cartridges (CBA, Bond Elut, 500-mg bedmass, 1-mL tube volume) were preconditioned with 1.0 mL acetonitrile and 1.0 mL 50 mM  $K_2HPO_4$  (pH 6.8). The sample was loaded and the cartridges washed twice with 1.0 mL 50 mM  $K_2HPO_4$  and twice with 1.0 mL of water. Elution was performed twice with 0.5 mL acetonitrile/ammonia (95:5, v/v). Samples were dried under a gentle stream of air, and dissolved in 200  $\mu$ L of mobile phase (0.1% formic acid/isopropyl alcohol, 60:40, v/v). Chromatography was performed isocratically without an analytical column, using a C18 guard column (2.1 mm x 12.5 mm, 5  $\mu$ m). The mobile phase consisted of a 0.1% formic acid/isopropyl alcohol (60:40, v/v), delivered at a flow rate of 0.25 mL/min. Using this method, inconsistent results (recovery, peak shape, retention time) and issues with the analytical approach (column pressure) proved to be a problem.

Several changes from published methods were instituted to the analytical protocol, primarily to reduce or eliminate precipitate formation and resolve the chromatography in order to improve analytical accuracy and precision, as shown in **Table 3.1**. These changes which included enhanced sample clean up via additional wash steps with acetonitrile and an ethyl acetate containing 2% formic acid prior to analyte elution; a change in elution solvent to avoid precipitate formation by using methanol and 2% formic acid as opposed to ammonia; final sample filtration to remove any precipitates; change in mobile phase; change in column; and use of gradient elution, with a flow rate of 0.35 mL/min. The mobile phase (organic) was changed to methanol from isopropyl alcohol, which seemed to reduce precipitate formation following sample injection into the LC system. Other changes included serum dilution with 50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.8) buffer solution, rather than water, to maintain a stable pH, and inclusion of a C18 analytical column (Agilent Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 µm) to improve analyte separation. Further information on the bison serum extraction method and validation is provided in **Chapter 4**.

**Table 3.1:** Changes in analytical conditions from published literature methods.

<b>Published Methods</b>	<b>Current Extraction &amp; Analytical Conditions</b>
0.5 mL serum + 0.5 mL water + internal standard.	0.5 mL serum + 0.5 mL buffer + 100 µL ACN. Centrifuge 21380 x g.
Condition SPEs: 1 mL ACN (x2) + 1 mL buffer (x2)	Condition SPEs: 1 mL ACN (x2) + 1 mL buffer (x2)
Load sample.	Load sample.
SPE Wash: 1 mL buffer (x2) 1 mL water (x2)	SPE Wash: 1 mL buffer (x2) 1 mL water (x4) 1 mL ACN (x4) 1 mL EA + 2% formic acid
Elute (x2): 0.5 mL ACN/ammonia (95:5, v/v)	Elute (x3): 1.0 mL MeOH + 2% formic acid
Concentrate under air.	Concentrate under nitrogen.
Dissolve in 200 µL mobile phase.	Dissolve in 200 µL MeOH + 300 µL 1% formic acid, then filter sample.
Isocratic elution.	Gradient elution.
C-18 guard column (2.1 x 12.5 mm, 5 µm).	C-18 analytical column (2.1 x 50 mm, 2.7 µm).
Mobile phase: 0.1% formic acid/isopropyl alcohol (60:40, v/v)	Mobile phase: A: 0.1% formic acid + 5 mM ammonium formate and B: MeOH
Flow rate: 0.25 mL/min	Flow rate: 0.35 mL/min

ACN = acetonitrile; buffer = 50 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6.8; EA = ethyl acetate; MeOH = methanol; SPEs = solid phase extraction cartridges.

### 3.2 Analytical Parameters

The instrument used was a Waters Alliance 2695 HPLC coupled to a Waters ZQ Micromass mass spectrometer, operated under positive electrospray ionization (ESI+) and monitored in selected ion monitoring (SIM) mode. The double charged ion  $[M+2H]^{+2}$ ,  $m/z$  403.9, was used for quantification of tulathromycin. Different aqueous mobile phase solutions were tested for the detection of tulathromycin in solution. The combination of 5 mM ammonium formate with 0.1% formic acid produced noticeably higher ionization of tulathromycin than when each solution was tested separately (5 mM ammonium formate at pH 3.9 or 0.1% formic acid).

### 3.3 Internal Standard

Using an internal standard in quantitative analysis is highly recommended. An internal standard is a known amount of compound that is added to the sample prior to extraction to aid in the quantification process (ADHS, 2013). The signal from the analyte is compared with the signal from the internal standard to determine how much analyte is present (ADHS, 2013; Aboul-Enein, 1998). Peak area ratios of analyte to internal standard are used for quantification. An internal standard is meant to correct for variations due to sample preparation (dilution, evaporation, degradation, recovery), since any loss of analyte during sample preparation can be paralleled by loss of the internal standard (ADHS, 2013; Aboul-Enein, 1998). An internal standard can also correct for variations due to instrumental parameters, such as injection volume, or fluctuations in detector response and ionization efficiency (ADHS, 2013).

The internal standard must be similar in analytical behaviour (similar chemical structure) to the analyte in order to mimic its behaviour. However, the behaviour of the internal standard in evaluating sample loss accurately is often overlooked (Aboul-Enein, 1998). The internal standard must not affect the target analyte, must produce unambiguous response in regards to the chromatographic detector system, and must be compounds that are not found in the sample (ADHS, 2013; Aboul-Enein, 1998). Bioanalytical data can be impaired if selection of an internal



standard is inappropriate (Mulvana, 2010). We chose the structural analogue, roxithromycin, which has been used as an internal standard for tulathromycin quantification (Huang et al., 2012; Scheuch et al., 2007). The addition of the ethyl acetate wash step during sample clean up however, resulted in the elution and loss of roxithromycin. Tulathromycin did not elute with ethyl acetate and we proceeded with our method development and validation without an internal standard. Despite the absence of the internal standard, careful sample preparation and extraction procedures and use of quality controls resulted in linear standard curves and highly consistent inter-assay accuracy and precision values.

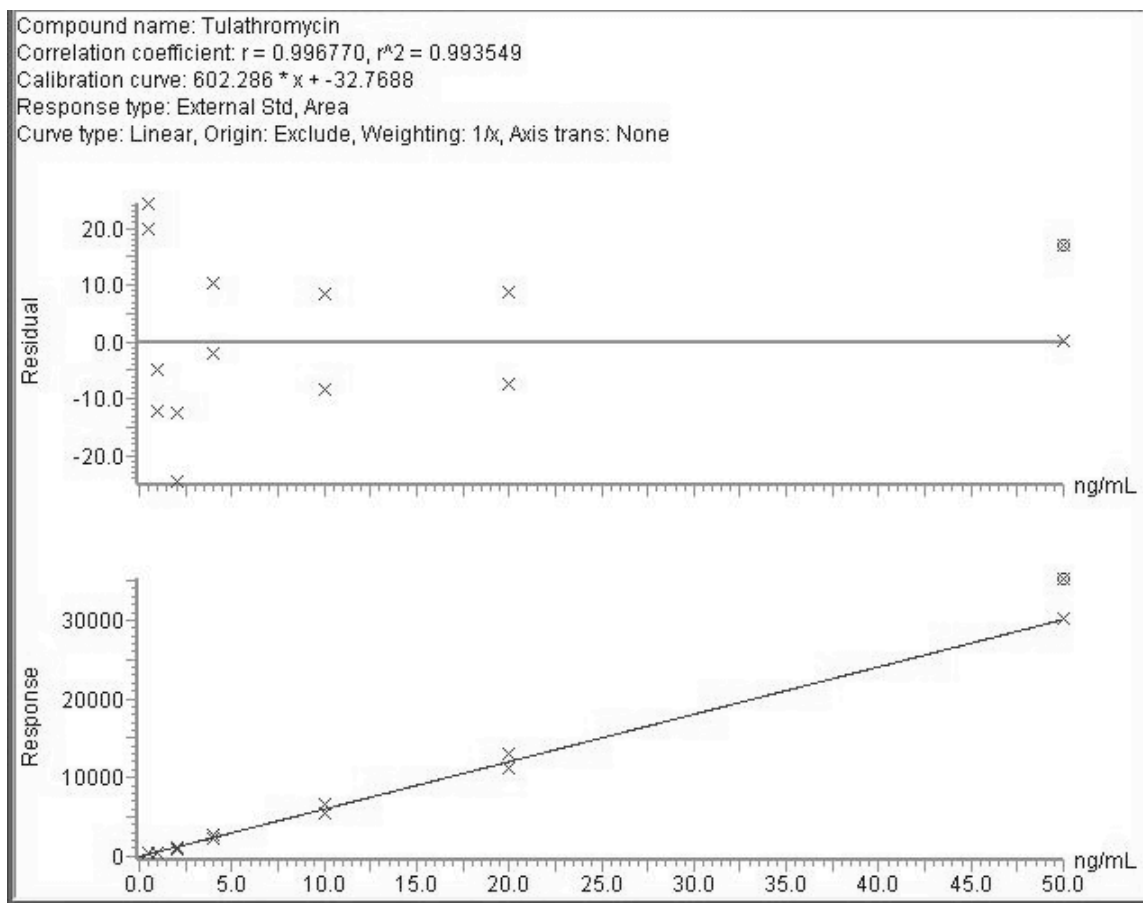
Each analytical run was set up in a specific order to ensure confidence in the quantified results. After instrument calibration, a system suitability check was done, which consisted of a mobile phase sample spiked with known amount of tulathromycin standard. The same sample was repeatedly injected into the instrument 5 times and the area for each compared, which were consistent, demonstrating the instrument was calibrated and working well. Following system suitability, the standard curve was injected, followed by the blank and quality control (QC) samples, consisting of two low, two middle, and two high concentration QCs. The extracted unknown samples were injected next, followed by the standard curve a second time, the QCs a second time, and the system suitability a second time. The differences between the beginning and end system suitability was <10%, and all QCs accuracy and precision were <15%.

### **3.4 Deer Serum Extraction Method**

The method developed and validated in bison serum was tested in deer serum, but similar results were not achieved. The high end of the calibration curve was lowered from 100 ng/mL to 50 ng/mL due to nonlinear responses beyond 50 ng/mL. A calibration curve in deer serum is shown in **Figure 3.2**. The acetonitrile precipitation step produced a coagulated mass and not a protein precipitate. This caused clogging of the SPE cartridges and an inconsistent extraction recovery and quantitation (accuracy, precision). To resolve this issue, samples were filtered in a 1.5 mL centrifugal filter tube, which produced a recovery of <20%. Extensive variability was observed between samples in precision and accuracy and generally, the method was overall inconsistent. We attempted to employ an internal standard and a number of different compounds were tested as an internal standard. Out of 13 macrolides tested, only gamithromycin appeared to

behave similarly to tulathromycin and was not lost in the ethyl acetate wash step. However, the new internal standard did not improve curve linearity or assay accuracy and precision.

To improve tulathromycin recovery in deer serum, acetonitrile, formic acid (0.1%, 1%), and hydrochloric acid (HCl) (0.012 M, 0.12 M) were used as the dilution solvent rather than the phosphate buffer. These solvents failed to increase recovery. Sample dilution (0.5 mL + 4.5 mL buffer) also failed to improve the extraction procedure. Sample precipitation with trifluoroacetic acid (TFA), formic acid, or 1 M HCl, resulted in extensive precipitate formation and poor sample recovery. Nonetheless, TFA worked best and different volumes of TFA were tested to optimize recovery. To 1.0 mL of serum, 100  $\mu$ L of acetonitrile and 20  $\mu$ L of TFA were added to each sample. Samples were centrifuged (21380 x g) and 0.5 mL transferred to a new 1.5 mL centrifuge tube and diluted with 0.5 mL of 50 mM  $K_2HPO_4$  (pH 6.8). Samples were centrifuged a second time before being loaded onto the SPE cartridge. The remaining extraction steps were the same as in the bison serum method. **Table 3.2** shows the method comparison between deer serum and bison serum. More information on the deer serum extraction method and validation is provided in **Chapter 5**.



**Figure 3.1:** Representative fortified tulathromycin linear calibration curve and residual plot in deer serum, with a weight of  $1/x$  and  $r^2$  value of 0.99.

**Table 3.2:** Comparison of the tulathromycin extraction method in bison and deer serum.

Bison Serum Extraction Method	Deer Serum Extraction Method
0.5 mL serum + 0.5 mL buffer + 100 $\mu$ L ACN. Centrifuge 21380 x g.	1.0 mL serum + 100 $\mu$ L ACN + 20 $\mu$ L TFA Centrifuge 21380 x g.
	Transfer 0.5 mL to new 1.5 mL centrifuge tube.
	Add 0.5 mL buffer. Centrifuge 21380 x g.
Condition SPEs: 1 mL ACN (x2) + 1 mL buffer (x2)	Condition SPEs: 1 mL ACN (x2) + 1 mL buffer (x2)
Load sample.	Load sample.
SPE Wash: 1 mL buffer (x2) 1 mL water (x4) 1 mL ACN (x4)	SPE Wash: 1 mL buffer (x2) 1 mL water (x4) 1 mL ACN (x4)

1 mL EA + 2% formic acid	1 mL EA + 2% formic acid
Elute (x3): 1 mL MeOH + 2% formic acid	Elute (x3): 1 mL MeOH + 2% formic acid
Concentrate under nitrogen.	Concentrate under nitrogen.
Dissolve in 200 $\mu$ L MeOH + 300 $\mu$ L 1% formic acid, then filter sample.	Dissolve in 200 $\mu$ L MeOH + 300 $\mu$ L 1% formic acid, then filter sample.
ACN = acetonitrile; buffer = 50 mM K <sub>2</sub> HPO <sub>4</sub> , pH 6.8; EA = ethyl acetate; MeOH = methanol; SPEs = solid phase extraction cartridges; TFA = trifluoroacetic acid.	

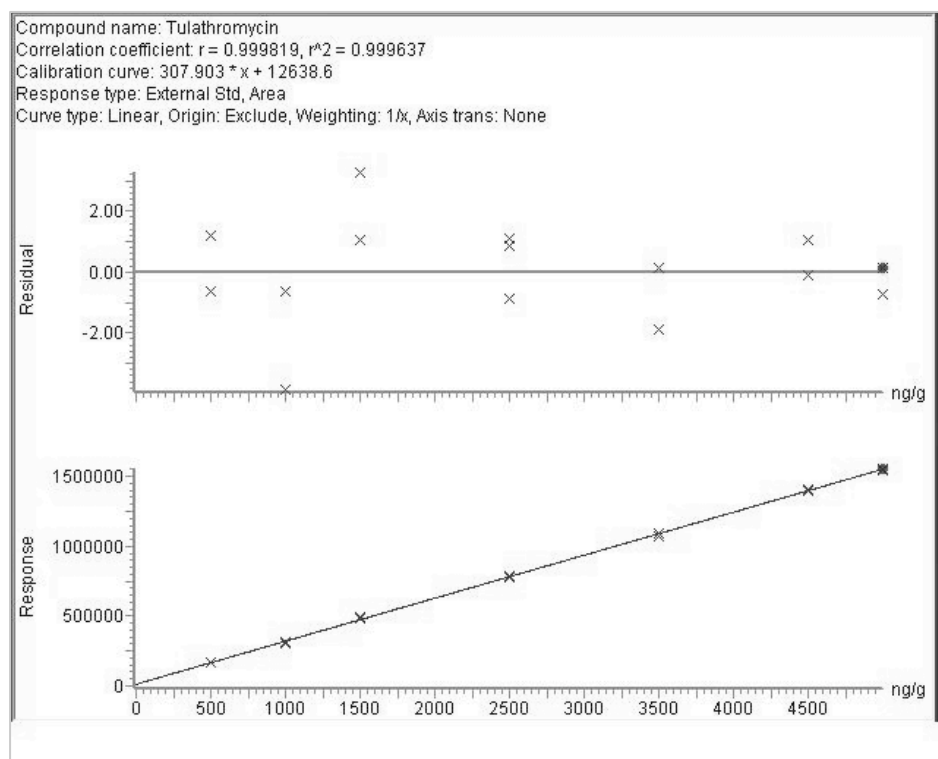
### 3.5 Deer Tissue Method Development

Preliminary method development was conducted in bovine muscle first, as there was limited availability of blank deer lung and muscle tissues. A number of different extraction solvents were tested, including acetonitrile, and a combination of acetonitrile with TFA, isopropyl alcohol, water, and buffer. Different molar strengths of K<sub>2</sub>HPO<sub>4</sub> buffer (50 mM and 0.1 M) were also tried, as was sodium chloride in the extraction step. Bond Elut-CBA (200 mg, 3 mL) and Nexus WCX (60 mg, 3 mL) extraction cartridges were also tested. After extraction and before sample clean up by solid-phase extraction, the extract supernatant was centrifuged and glass wool was added to each SPE cartridge after conditioning to remove particulates and prevent the cartridges from clogging up. The amount of tissue used was switched from 5.0 g to 2.0 g to accommodate the amount of blank tissue that was available for the calibration curves and quality control samples. Wash steps were added as the method was adjusted to clean up extracts as best as possible.

To establish an appropriate calibration curve range, a subset of samples used in the deer withdrawal study were extracted and quantified to provide an indication of what concentrations to expect in the study samples. Calibration curves were then selected to fall within the expected values for tulathromycin concentration in deer lung (500 to 5000 ng/g) and muscle (100 to 2500 ng/g) tissues. A sample calibration curve in deer lung is shown in **Figure 3.3**. Once it was established tissues would contain high concentrations of drug, the method was adjusted from evaporating the eluates, as in the serum extractions, to dilution with water.

To 2.0 g ( $\pm$  0.05 g) of tissue in a 50 mL centrifuge tube, 5.0 mL of acetonitrile and 5.0 mL of 50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.8) were added. Samples were vortex mixed well, shaken for 15 min, and centrifuged (3917 x g) for 10 min. The supernatant was transferred to a new 50 mL tube and an additional 2.5 mL acetonitrile and 2.5 mL 50 mM K<sub>2</sub>HPO<sub>4</sub> were added to the tissue pellet

which was then vortex mixed, shaken, and centrifuged a second time. The supernatant was transferred to the same tube containing the first extract. Supernatants were centrifuged ( $3917 \times g$ ) to remove any particulates before solid phase extraction. Cartridges (Bond Elut-CBA, 200 mg, 3 mL) were preconditioned with 3.0 mL ACN and 3.0 mL buffer. Glass wool was added to each extraction cartridge to remove tissue particle or debris. After the sample was loaded, the cartridges were washed with 3.0 mL buffer (x2), removing the glass wool after the first wash, followed by 3.0 mL water (x3), 3.0 mL acetonitrile, 3.0 mL ethyl acetate, 3.0 mL hexane, and 3.0 mL ethyl acetate containing 2% formic acid. The cartridges were dried for 15 min under vacuum to remove any unwanted solvent before elution with 1.0 mL of methanol containing 2% formic acid (x3) into 15 mL centrifuge tubes. The total volume was adjusted to 10.0 mL with Millipore water. Each tube was mixed and 500  $\mu$ L subsample transferred to an LC filter vial before LC-MS analysis. The extraction method used in deer muscle and lung tissue is presented in **Table 3.3**. Further information regarding analytical method validation in deer muscle and lung is presented in **Chapter 5**.



**Figure 3.2:** Representative fortified deer lung linear calibration curve and residual plot for tulathromycin, with a weight of  $1/x$  and an  $r^2$  value of 0.99.

**Table 3.3:** Tulathromycin tissue extraction method in deer muscle and lung.

2.0 g ( $\pm$ 0.05 g) ground tissue + 5.0 mL ACN + 5.0 mL buffer.
Vortex 1 min, shake 15 min, centrifuge (3917 x g) 10 min.
Transfer supernatant to new 50 mL centrifuge tube.
Add 2.5 mL ACN + 2.5 mL buffer to tissue pellet.
Vortex, shake, centrifuge.
Combine both supernatants and centrifuge.
Condition SPE cartridges: 3.0 mL acetonitrile + 3.0 mL buffer. Add glass wool to each cartridge.
Load sample.
SPE Wash: 3.0 mL buffer (x2), remove glass wool after 1 <sup>st</sup> wash 3.0 mL water (x3) 3.0 mL ACN 3.0 mL EA 3.0 mL Hexane 3.0 mL EA + 2% formic acid
Dry cartridges 15 min under vacuum.
Elute (x3): 1.0 mL MeOH + 2% formic acid.
Adjust volume to 10.0 mL with Millipore water.
Mix and filter 500 $\mu$ L subsample for LC-MS analysis.

ACN = acetonitrile; buffer = 50 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6.8; EA = ethyl acetate; MeOH = methanol;  
SPEs = solid phase extraction cartridges.

## 4. TULATHROMYCIN PHARMACOKINETICS IN NORTH-AMERICAN BISON (*BISON BISON*) USING MASS SPECTROMETRIC ANALYSIS

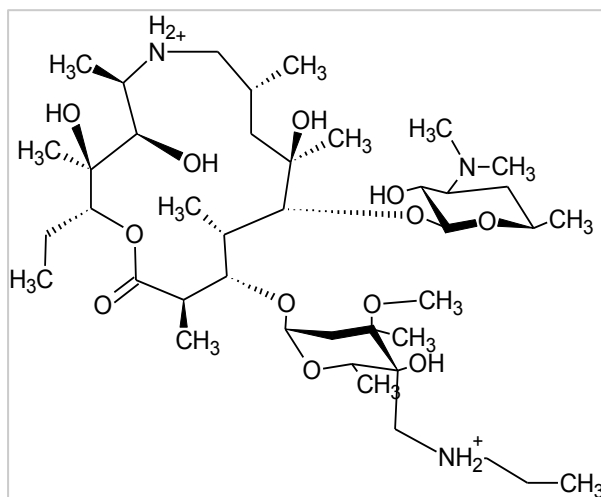
### 4.1 Introduction

Limited available information on drug dosing and withdrawal times in bison species makes treating respiratory disease challenging for veterinarians and producers. Since no antimicrobial drugs are approved in North America for use in this species (Woodbury, 2012), bison are typically treated with antibiotics approved for use in cattle and used at the recommended cattle dose. This practice requires an assumption that bison share similar drug absorption and disposition characteristics with cattle. However, significant differences between species potentially lead to markedly different peak and duration of drug levels among species (Toutain et al., 2010). Consequently, dosage regimens in the species for which the drug is licensed in (i.e. cattle) may be inappropriate for bison. Bison industries continue to prosper in today's economy (NBA, 2013). With inevitable production intensification, diseases, including respiratory infections (ex. *Mycoplasma bovis*), are becoming a leading cause of death in bison (Janardhan et al., 2010; Dyer et al., 2008). This identifies a paramount need to determine species-specific effective drug therapy regimens to mitigate the morbidity and mortality associated with such diseases.

Tulathromycin (**Figure 4.1**; 806.23 g/mol;  $C_{41}H_{79}N_3O_{12}$ ) is a macrolide antibiotic approved for use in bovine and swine bacterial respiratory disease (Drugs.com, 2013; APVMA, 2007; Evans 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Studies demonstrate high efficacy of tulathromycin for the treatment and prevention of bovine and swine respiratory disease when administered as a single dose (Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Nowakowski et al., 2004). In these species, tulathromycin undergoes rapid absorption ( $C_{max}$  <1 hour) and extensive distribution to tissues (APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Elimination half-lives typically range between 3 to 5 days (Drugs.com, 2013; Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Gáler et al., 2004). Metabolism is minimal and tulathromycin is eliminated primarily as unchanged parent drug by biliary excretion (Drugs.com, 2013; APMVA, 2007; Evans, 2005; Nowakowski et al., 2004).

Often, multiple injections of macrolides, such as erythromycin and tylosin, are needed to ensure effective therapeutic outcomes (Young et al., 2010; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004). Tulathromycin, however, is formulated as a long acting, single-dose injection therapy (APVMA, 2007; Evans, 2005; Gáler et al., 2004; Nowakowski et al., 2004; Benchaoui et al., 2004). Furthermore, its chemical structure consists of three amine groups, which allows for greater penetration into tissues and continued activity in the lung after a single injection (APVMA, 2007; Evans, 2005; FDA/CVM, 2004; Gáler et al., 2004). Such characteristics are desirable in the bison industry where the difficulty associated with bison handling is a critical barrier to effective therapy. Today, tulathromycin is used off-label in bison species; however, a review of the published literature failed to find reference to the study of this antibiotic in this species.

In aqueous solution, tulathromycin is a mixture of two equilibrating isomers, tulathromycin A (CP-472,295) and B (CP-547,272), in a 9:1 ratio. Consequently, quantitative assays focus on isomer A (Huang et al., 2012; APVMA, 2007; Scheuch et al., 2007; Evans, 2005; Gáler et al., 2004). In our study, an LC-MS method was developed for the quantification of tulathromycin A in bison serum to investigate the pharmacokinetics of tulathromycin in bison following a single 2.5 mg/kg bw subcutaneous injection. We then compared our PK parameter estimates to cattle parameters to predict the adequacy of the recommended cattle dose in bison.



**Figure 4.1:** The molecular structure of tulathromycin A (806.23 g/mol;  $C_{41}H_{79}N_3O_{12}$ ). The proposed double charged ion,  $m/z$  403.9, is shown with ionization most likely localized at the secondary amines and was used for quantitative analysis in bison serum.



## 4.2 Materials and Methods

### 4.2.1 Chemicals

Tulathromycin analytical standard was a kind gift from Pfizer Inc. (Groton, CT). The commercially available form of tulathromycin, *Draxxin*, containing 100 mg of tulathromycin/mL (Zoetis Canada, Kirkland, QC), was administered to bison used in the study. Potassium phosphate dibasic anhydrous buffer,  $K_2HPO_4$ , (Certified, ACS) and ammonium formate were purchased from Fisher Scientific (Ottawa, ON). Formic acid (98% GR ACS) was purchased from VWR (Edmonton, AB). Acetonitrile, methanol, and ethyl acetate, all HPLC grade, were purchased from Caledon laboratories (Georgetown, ON). Millipore water was prepared by a Milli-Q Advantage water purification system (Fisher Scientific, Ottawa, ON).

### 4.2.2 Preparation of Standard Solutions

A 1.0 mg/mL (1000  $\mu$ g/mL) standard stock solution of tulathromycin was prepared by dissolving the tulathromycin standard in 10 mL of acetonitrile. Working standard solutions of 10  $\mu$ g/mL and 100 ng/mL were prepared by diluting 100  $\mu$ L of the 1000  $\mu$ g/mL stock solution, or 100  $\mu$ L of the 10  $\mu$ g/mL working standard solution into 10 mL acetonitrile, respectively. Stock standard was prepared quarterly and stored at -20°C, while working standards were made fresh before each analysis.

### 4.2.3 Bison Pharmacokinetic Study

Ten wood bison (*Bison bison athabasca*, female, 4-7 years) were used in the study. The animals were housed at the Specialized Livestock Research Facility, University of Saskatchewan. Animals were fed on pasture forage during the summer months, and baled forage with mineral supplements in the winter months. Fresh water was available at all times. Routine preventative health care was provided consisting of annual application of anthelmintic (*Ivomec*; Merial Canada Inc., Baie-D'Urfe, QC) and a multivalent Clostridial vaccine (*Covexin 8*, Schering-Plough Canada Inc., Kirkland, QC). Animals had no known history of macrolide exposure. This

study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Animals were given a single 2.5 mg/kg subcutaneous injection of *Draxxin* (Zoetis Canada, Kirkland, QC) in the lateral neck. This dose is the therapeutic dose approved for cattle. Blood samples were collected in 10 mL serum (red top) vacutainer tubes by jugular venipuncture at 1, 2, 4, 6, 12 hours, and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, 19, 21, 23, and 25 days post-injection. Additionally, a blood sample was taken from each animal prior to dosing for use as a blank control. Animals were observed daily for general health and clinical observations were made prior to injection and at collection times post-dose. Serum was separated by centrifugation (Eppendorf 5804 R Centrifuge) at 1500 x g for 10 min, transferred to microcentrifuge tubes, and stored at -80°C until analysis.

#### **4.2.4 Sample Preparation**

Drug free (blank control) bison blood samples were obtained from each of the ten bison used in the study, plus one additional animal during slaughter. Samples were centrifuged, serum transferred into polypropylene centrifuge tubes, and stored at -20°C. Drug-free serum samples (0.5 mL) were spiked with working standard solutions of tulathromycin (standard plus acetonitrile up to 100 µL) to prepare the calibration curve and quality control samples prior to extraction.

To 0.5 mL serum sample, 100 µL of acetonitrile and 0.5 mL of a 50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.8) solution were added. The samples were vortex mixed and centrifuged (Refrigerated Microcentrifuge Z 216 MK, Hermle Labor Technik) at 21380 x g for 15 min at 5°C. Solid phase extraction cartridges (Bond Elut –CBA, 50-mg bedmass, 1-mL tube volume, Agilent Technologies, Ottawa, ON) were preconditioned twice with 1.0 mL acetonitrile and twice with 1.0 mL of the 50 mM K<sub>2</sub>HPO<sub>4</sub> buffer solution. The samples were loaded and the cartridges washed thoroughly with 1.0 mL K<sub>2</sub>HPO<sub>4</sub> (x2), 1.0 mL Millipore water (x4), 1.0 mL acetonitrile (x4), and 1.0 mL ethyl acetate containing 2% formic acid (x2). The cartridges were dried under vacuum for 7 min to allow removal of excess solvent before elution with 1.0 mL methanol containing 2% formic acid, 3 times (total volume 3.0 mL). Eluates were evaporated to dryness under nitrogen in a 40°C water bath and dissolved in 200 µL methanol and 300 µL of 1% formic

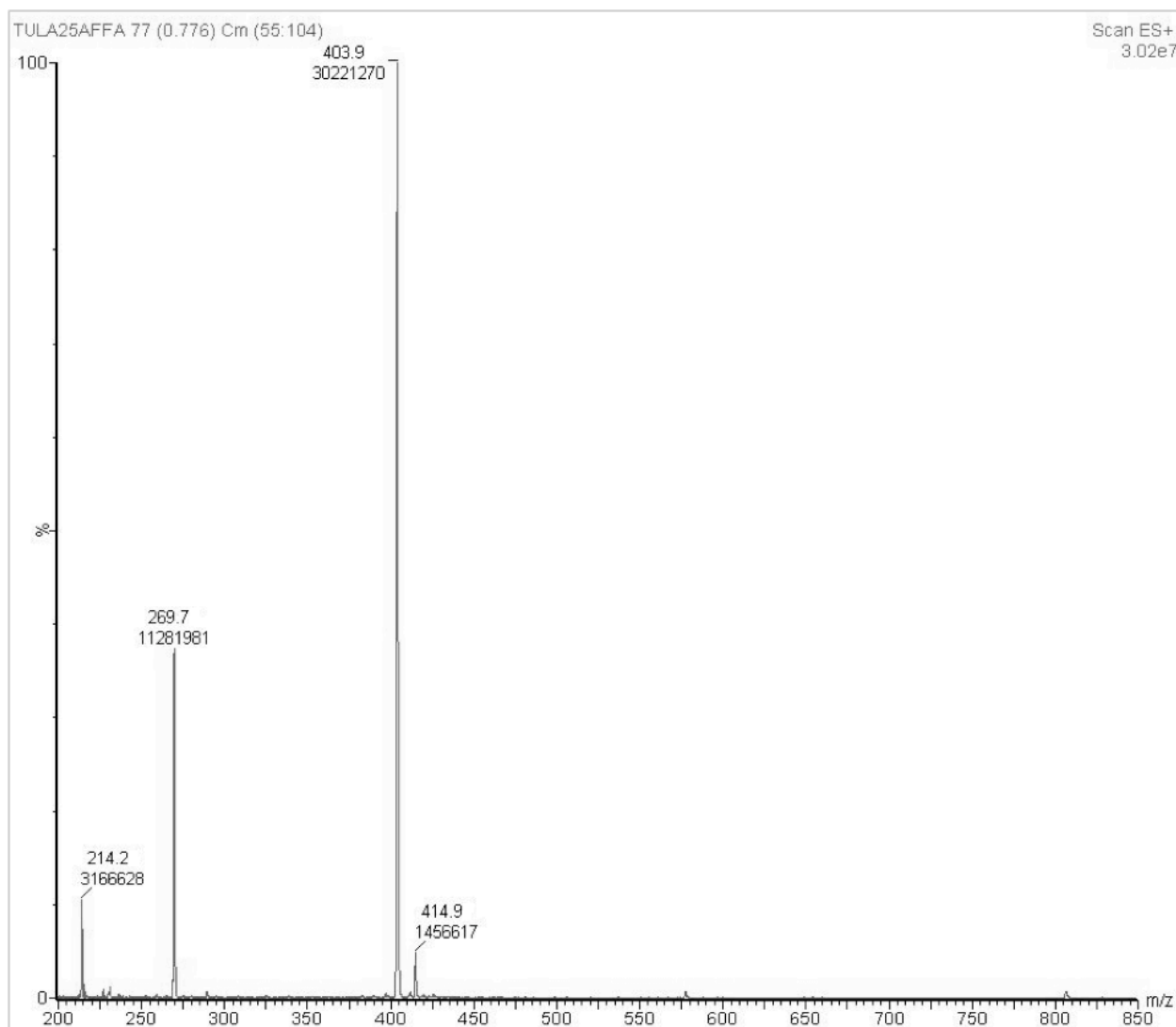
acid, vortex mixed, and sonicated for 15 min before being transferred to an LC filter vial (Whatman Mini-Uniprep Syringeless Filter, PTFE, 0.2  $\mu$ m; VWR, Edmonton, AB). A 5  $\mu$ L sample was injected for LC-MS analysis.

#### 4.2.5 Chromatography and Mass Spectrometry

A Waters Alliance 2695 high performance liquid chromatography (HPLC) coupled to a Waters Micromass ZQ mass spectrometer (Waters Corporation, Milford, MA) was used for quantification of tulathromycin. The HPLC system consisted of a pump, degasser, autosampler (set at 10°C), and column heater (set at 50°C). Chromatography was performed by gradient elution using a Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7  $\mu$ m, Agilent Technologies, Ottawa, ON), with a 0.5  $\mu$ m Frit (Canadian Life Science, Edmonton, AB) attached to each end. The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid (A), and 100% methanol (B), delivered at a flow rate of 0.35 mL/min. The gradient was 80% A for 1.0 min, ramped to 25% A until 6.5 min, then to 5% A until 9.0 min. At 9.2 min, the conditions were returned to initial conditions (80% A) and held until 15 min to allow for column re-equilibration. Total run time was 15 min with analyte elution occurring at 4.2 min.

Electrospray ionization (ESI) was used in positive ionization mode. Compound optimization was performed by infusion using a model 11 Plus syringe pump (Waters Corporation, Milford, MA). The double charge ion  $[M+2H]^{+2}$  at  $m/z$  403.9 was monitored in selected ion mode (SIM) and used for quantification (**Figure 4.1**). The positive ion ESI-MS spectrum of tulathromycin is shown in **Figure 4.2**. Optimal instrument parameters are presented in **Table 4.1**.

MassLynx version 4.1 (Waters Corporation, Milford, MA) was used to acquire and quantify the data, while Microsoft Office Excel 2007 (Microsoft Corporation) and Minitab version 12.1 (Minitab Inc., 1998) were used for statistical evaluation.



**Figure 4.2:** Positive ion electrospray mass spectra of tulathromycin obtained on a Waters Micromass ZQ mass spectrometer. An ion carrying a double charge was observed at  $m/z$  403.9.

**Table 4.1:** Optimal instrument parameters for the determination of tulathromycin in bison serum using positive ion electrospray ionization. Selected ion monitoring (SIM) mode was used using a Waters Micromass ZQ mass spectrometer instrument.

Variable	Value
Cone gas	50 L/hr
Desolvation gas	1100 L/hr
Capillary voltage	1.70 kV
Cone voltage	25 V
Source temp.	120°C
Desolvation temp.	450°C

#### 4.2.6 Validation Procedures

The method was validated according to international guidelines (CDER, 2001). Validation parameters included selectivity, calibration curve linearity, accuracy and precision, recovery, stability, and dilution integrity.

Selectivity was verified using blank bison serum compared to serum spiked with tulathromycin in replicates of five. The calibration curve consisted of eight standards between 0.8 ng/mL and 100 ng/mL, plus a blank. Six quality control samples, two of each at low (1.5 ng/mL), middle (15 ng/mL), and high (80 ng/mL) concentrations of tulathromycin, were also processed with each curve. For accuracy and precision, quality control (QC) samples were prepared using blank samples spiked with standard solutions at a low (LQC), middle (MQC), and high (HQC) concentration range. Intra- and inter-day precision and accuracy were determined by analysis of six replicates of samples at three different concentrations on three different days. Recovery of tulathromycin was assessed by comparing calibration samples spiked with tulathromycin before extraction (matrix fortified) to those spiked after extraction (matrix matched). Stability of sample extracts was assessed by analyzing samples on the day they were extracted and again one week after extraction. Samples extracted on day one were stored at 5°C for one week, re-analyzed and compared to day one. Since some samples were anticipated to be greater than the standard curve calibration range (>100 ng/mL), dilution integrity was determined to ensure accuracy of diluted samples. Dilution integrity was validated by extracting known amounts of spiked samples and diluting them 2-fold, 5-fold, and 10-fold in replicates of at least two.

#### 4.2.7 Pharmacokinetic Data Analysis

A non-compartmental approach was used to analyze the serum concentration versus time data. Pharmacokinetic (PK) parameters were estimated for each individual bison serum concentration-time curve using GraphPad Prism software (Version 5.0, San Diego, California). The PK parameters estimated from each individual animal included the log-linear terminal rate constant ( $k$ ), area under the serum concentration-time curve extrapolated to infinity ( $AUC_{0-\infty}$ ), area under the first moment serum concentration-time curve extrapolated to infinity ( $AUMC_{0-\infty}$ ), half life ( $t_{1/2}$ ), mean residence time (MRT), maximum serum concentration ( $C_{max}$ ), and the time to  $C_{max}$  ( $t_{max}$ ).  $C_{max}$  and  $t_{max}$  were taken visually from the serum concentration-time curves. The

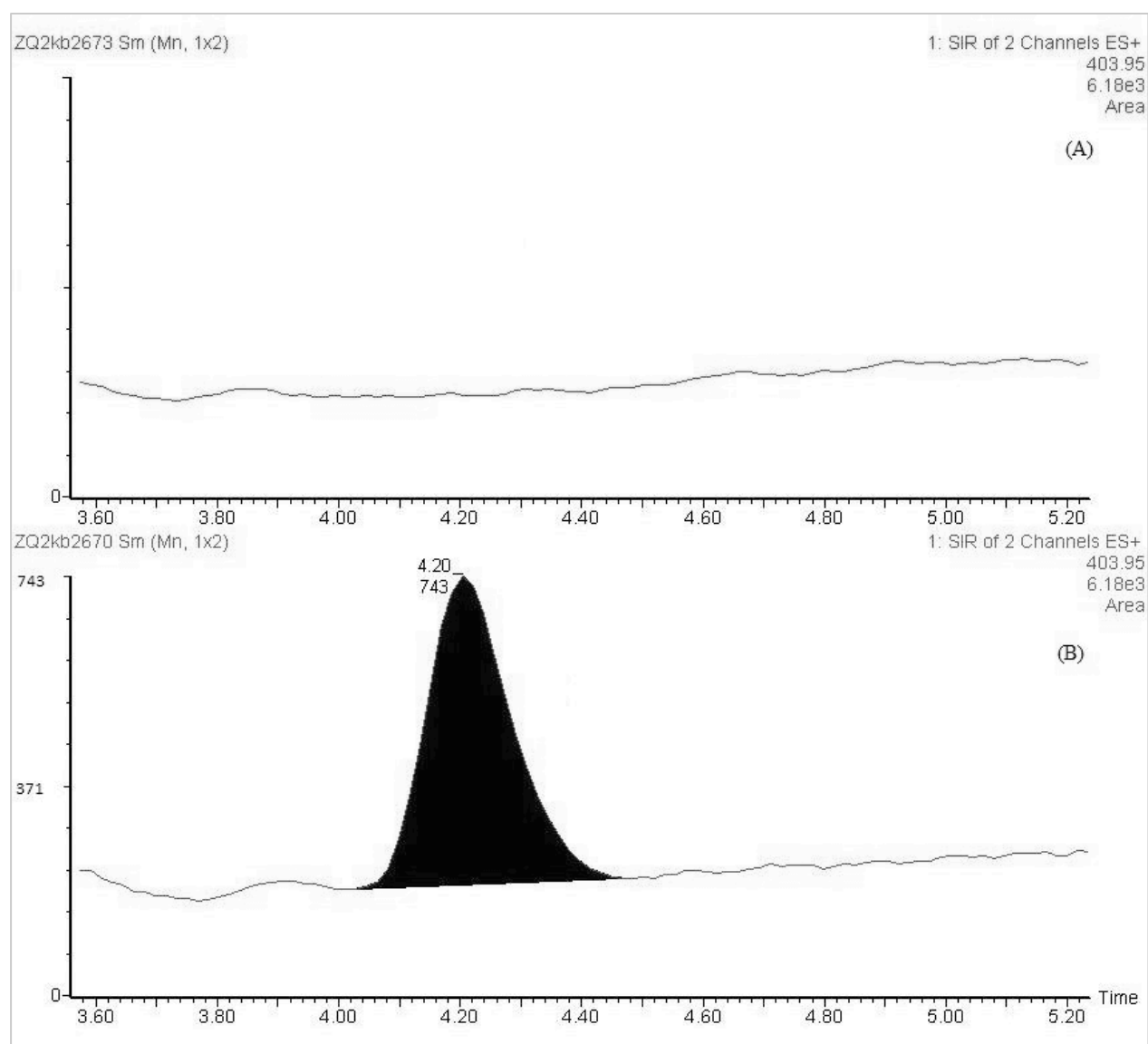
$AUC_{0-inf}$  and  $AUMC_{0-inf}$  values were calculated using the trapezoidal rule-extrapolation method (assessed up to the last sampling time and then extrapolated to infinity).  $k$  was calculated from the slope of the terminal portion of the natural logarithm concentration versus time plot, and half-life was calculated as the ratio of 0.693 to  $k$ . The apparent clearance ( $Cl/F$ ) and apparent volume of distribution ( $V_d/F$ ) were also calculated, as  $dose/AUC_{0-inf}$  and  $dose/AUC_{0-inf} * k$ , respectively. The MRT was calculated from the ratio of  $AUMC_{0-inf}$  to  $AUC_{0-inf}$ . PK parameter estimates were calculated for each individual animal and reported as the mean and standard deviation of all individual animals.

## 4.3 Results

### 4.3.1 LC-MS Assay Validation

For LC-MS assay validation, selectivity was first tested by comparing blank bison serum to blank serum spiked with tulathromycin in replicates of five. **Figure 4.3** shows a representative chromatogram of blank matrix fortified sample and a 0.8 ng/mL tulathromycin spiked in bison serum. Both chromatograms were obtained after extraction. LC-MS detection of the double charged ion,  $m/z$  403.9, was specific to tulathromycin.

The calibration curve was linear from 0.8 ng/mL to 100 ng/mL ( $r^2 > 0.99$ ). The limit of detection (LOD) was statistically calculated as 0.3 ng/mL, and the limit of quantitation (LOQ) was 0.8 ng/mL. A weight of  $1/x$  was applied to the calibration curve. Recovery ranged from 76% to 80%. Matrix enhancement was also noted, where analytical results for extracted samples (matrix fortified) were compared to chemical standards that represent 100% recovery. In other words, bison serum increased the signal for the analyte resulting in ion enhancement.



**Figure 4.3:** Representative LC-MS chromatogram of a blank extract of bison serum (A) and a blank bison serum fortified with 0.8 ng/mL tulathromycin (B).

Intra- and inter-day accuracy and precision were determined by analysis of six replicates of quality control (QC) samples at three different concentrations on three different days. All values for the QCs fell within the acceptable criteria of  $\pm 15\%$ . Accuracy and precision values are given in **Table 4.2**.

Extracts were stable for at least one week when stored at 5°C. Freeze-thaw stability was not evaluated. Stock standards were more stable in acetonitrile, as compared to methanol, and are stable for at least four months when stored at -20°C.

Dilution integrity was validated by extracting known amounts of spiked samples followed by 2-fold, 5-fold, and 10-fold dilution with blank extract matrix in replicates of at least two. The determined values were very close to the expected values, as accuracy and precision were <15%.

**Table 4.2:** Inter- and intra-accuracy and precision data for tulathromycin in bison serum.

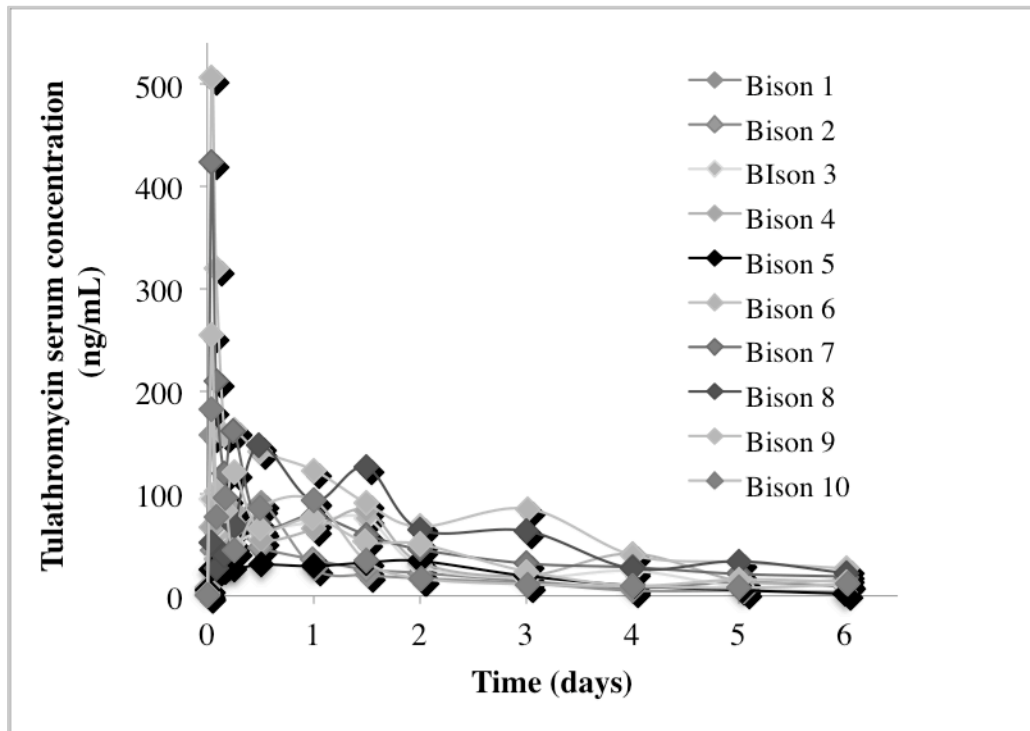
	Intra-Day:		Inter-Day:	
QC Sample	Precision (%) (n=6)	Accuracy (%) (n=6)	Precision (%) (n=18)	Accuracy (%) (n=18)
LQC (1.5 ng/mL)				
Day 1	7.8	7.8	5.9	10.1
Day 2	2.6	8.4		
Day 3	3.2	14.1		
MQC (15 ng/mL)				
Day 1	11.5	-9.2	7.8	-6.3
Day 2	4.2	-5.4		
Day 3	6.0	-4.3		
HQC (80 ng/mL)				
Day 1	12.7	2.7	7.7	1.1
Day 2	3.4	-1.2		
Day 3	4.6	1.9		

### 4.3.2 Tulathromycin Pharmacokinetics in Bison

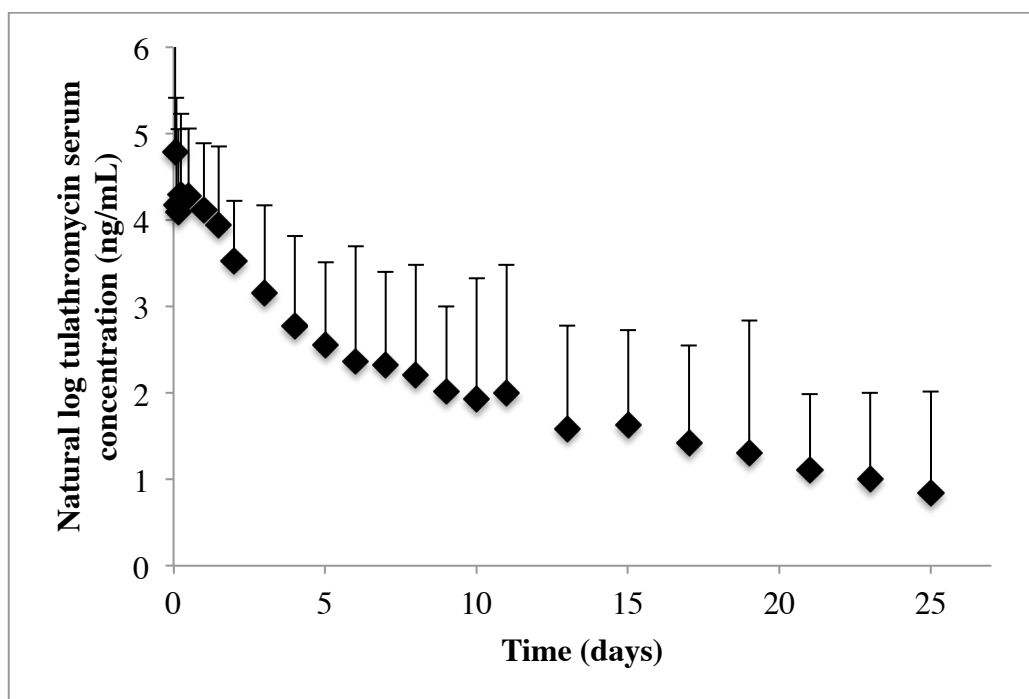
Serum concentration-time profiles of tulathromycin following a 2.5 mg/kg bw subcutaneous dose in the ten bison are shown in **Figures 4.4 and 4.5**. These figures demonstrate extensive interindividual variation in tulathromycin concentration-time profiles following a single 2.5 mg/kg bw subcutaneous injection. No adverse effects were observed in the present study using the commercial formulation of tulathromycin. **Table 4.3** reports the mean and standard deviation of each PK parameter estimate. Maximal serum concentrations ( $C_{\max}$ ) ranged from 30 to 506 ng/mL with a mean  $C_{\max}$  of 195 ng/mL. The majority of animals reached maximal serum concentration within 1 hour (median of 1.04 hours) after dosing ( $t_{\max}$ ), with four of the ten bison reaching  $C_{\max}$  after 1 hour (two animals at 6 hours and two at 12 hours).  $AUC_{0-\infty}$  values were between 3582 and 19339 ng\*hr/mL, with a mean  $AUC_{0-\infty}$  of 9341 ng\*hr/mL. The apparent volume of distribution ( $V_d/F$ ) following subcutaneous injection ranged between 34 and 175 L/kg.



Half-lives ranged between 173 and 312 hours (7 and 13 days), with a mean value of 214 hours (8.9 days). Mean residence time was 208 hours.



**Figure 4.4:** Tulathromycin serum concentrations of ten individual female bison following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* over time. The data has been truncated, showing data up to 6 days to give a better indication of variability and AUC.



**Figure 4.5:** Natural logarithmic plot of mean and standard deviation tulathromycin concentrations over time, following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* to ten female bison.

**Table 4.3:** Mean and standard deviation (SD) of each PK parameter following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* to ten female bison.

PK parameter	Mean (n=10)	SD
$k$ ( $h^{-1}$ )	0.0033	0.0006
$AUC_{0-inf}$ (ng*h/mL)	9341	5087
$C_{max}$ (ng/mL)	195	157
$t_{max}$ (h)	4.2*	4.6
$Cl/F$ (L/h/kg)	0.4	0.2
$V_d/F$ (L/kg)	111	66
$t_{1/2}$ (h)	214	43
$AUMC_{0-inf}$ ( $\mu g \cdot h^2/mL$ )	1985	1132
MRT (h)	208	40
*Median = 1.04 h		

#### 4.4 Discussion

To the best of our knowledge, we are the first to report the pharmacokinetics of tulathromycin in bison following a dosage regimen recommended for cattle. Tulathromycin reached maximal serum concentrations within 1 hour after subcutaneous injection, consistent with values reported in cattle (Gáler et al., 2004; Nowakowski et al., 2004). The mean  $C_{\max}$  (195 ng/mL) was lower in bison compared to cattle reported at 300 ng/mL (Gáler et al., 2004) and 500 ng/mL (Nowakowski et al., 2004). The mean bison  $AUC_{0-\infty}$  (9341 ng\*hr/mL) was also lower compared to cattle at 18700 ng\*hr/mL (Nowakowski et al., 2004). The apparent volume of distribution in bison (111 L/kg) was much larger than that reported in cattle at 11 L/kg (Nowakowski et al., 2004). The half-life of tulathromycin (214 hours) also was much longer compared to cattle reported as 90 hours (Gáler et al., 2004) and 110 hours (Nowakowski et al., 2004). Interestingly, tulathromycin pharmacokinetic characteristics reported in other species such as swine (Huang et al., 2012; Benchaoui et al., 2004), goats (Young et al., 2010), and foals (Scheuch et al., 2007), tend to exhibit similar values to cattle.

Whether tulathromycin exhibits greater tissue and lung penetration in bison is difficult to conclude based upon the collective pharmacokinetic parameter estimates obtained in this study. The bioavailable dose is an important determinant of both AUC and  $C_{\max}$  values. As compared to cattle, tulathromycin AUC and  $C_{\max}$  values were almost two-fold less in bison. This suggests that bioavailability following subcutaneous injection is lower in bison relative to cattle and other species. On the other hand, systemic clearance also determines AUC and  $C_{\max}$  values, and more rapid elimination kinetics in bison may explain the lower AUC and  $C_{\max}$  values in bison relative to other species. Half-life depends on both systemic clearance and volume of distribution. The apparent volume of distribution of tulathromycin was much greater in bison relative to cattle. However, without knowledge of tulathromycin bioavailability following subcutaneous injection, the larger apparent volume of distribution may simply reflect lower bioavailability in bison. The longer half-life of tulathromycin in bison seemingly contradicts the possibility of faster elimination kinetics; however, the concentration-time profiles in bison may represent flip-flop kinetics. Absorption kinetics may become the rate limiting process governing the terminal natural logarithmic concentration-time profiles with slow release kinetics of tulathromycin from the subcutaneous injection sites. Collectively, the pharmacokinetic characteristics of tulathromycin in bison do not support a greater penetration of tulathromycin into tissues, in particular, the lung.

Tissue distribution studies are needed to adequately assess pharmacokinetic characteristics and to determine whether the 2.5 mg/kg bw subcutaneous dose is adequate for use in bison.

Although the pharmacodynamics (PD) of tulathromycin in bison was not part of the present study, both PK and PD properties are important in determining antimicrobial efficacy (Evans, 2005; Zhanel et al., 2001). Older PD studies using macrolides suggest the time above minimum inhibitory concentration ( $T > MIC$ ) corresponds with clinical efficacy. However, newer macrolides (azithromycin) associate AUC/MIC, or total drug exposure to the pathogen, to be more predictive (Evans, 2005; Kilgore et al., 2005; Benchaoui et al., 2004; Nowakowski et al., 2004; Zhanel et al., 2001; Nightingale, 1997), and relying exclusively on time-dependent activity (time drug concentration is above MIC) fails to adequately predict the observed efficacy for macrolides (Evans, 2005; Kilgore et al., 2005). Newer macrolides achieve prolonged half-life in the target tissue allowing for persistent exposure of pathogens to the drug (Kilgore et al., 2005; Nowakowski et al., 2004). Generally, macrolide concentrations in plasma are much lower than those in tissues and the concentration of drug in plasma may even be below the MIC of the pathogen in which good clinical efficacy is well established (Evans, 2005; Nowakowski et al., 2004). This suggests the ratio of AUC:MIC may be more useful than  $T > MIC$ , as this measure incorporates both time and concentration elements (Evans, 2005; Kilgore et al., 2005). The  $AUC_{0-inf}$  was 2-fold lower in bison relative to reported values in cattle possibly suggesting the 2.5 mg/kg bw subcutaneous dose might exhibit poor clinical efficacy in bison.

No reported data are available for MIC values for tulathromycin against bison respiratory pathogens. The  $MIC_{90}$  (lowest concentration inhibiting 90% of isolates) values for tulathromycin against common respiratory pathogens in cattle are: *M. haemolytica* 2 µg/mL, *P. multocida* 1 µg/mL, *H. somni* 4 µg/mL and *M. bovis* 1 µg/mL (Evans, 2005; Kilgore, 2005). Tulathromycin concentrations in plasma for cattle (300 to 500 ng/mL or 0.3 to 0.5 µg/mL) are well below the MIC value for these pathogens, which would indicate poor tulathromycin efficacy if relying on plasma data alone. In general, the new macrolides including tulathromycin have unique pharmacokinetic properties and serum or plasma concentrations are poor predictors of antibacterial efficacy (Zhanel, 2001; Nightingale, 1997). Assuming MIC values are similar to those for pathogens in bison, a tulathromycin  $C_{max}$  in bison (195 ng/mL or 0.195 µg/mL) is well below the MIC. Given the poor relationship between antimicrobial efficacy and plasma or serum concentrations, evaluation of tissue concentrations at the site of infection are necessary in

understanding the PD of tulathromycin in bison (Benchoui et al., 2004; Nowakowski et al., 2004; Nightingale, 1997).

In cattle, tulathromycin rapidly distributes from plasma to tissue and lung AUC values become a better PD predictor of clinical efficacy than plasma AUC values. The lung half-life of tulathromycin in cattle is 184 hours (8 days), significantly longer than the plasma half-life at 90 hours (Nowakowski et al., 2004; Evans, 2005). Furthermore, the maximal lung concentrations in cattle are 4100 ng/g (4.1 µg/g), which is well over the MIC<sub>90</sub> values for bacterial pathogens in cattle. The high tissue concentrations and prolonged residence time contribute to the clinical efficacy of a single tulathromycin dose in cattle. Whether lung half-life and concentration is adequate in bison is not known. Studies are needed to evaluate MIC<sub>90</sub> for respiratory pathogens in bison, specifically *Mycoplasma bovis*, as well as lung tissue distribution studies to determine the clinical efficacy of tulathromycin in bison.

#### **4.5 Conclusion**

An LC-MS method was developed and validated for tulathromycin in bison serum following international criteria for bioanalytical method validation. In bison, a single 2.5 mg/kg bw subcutaneous injection of tulathromycin achieved maximum serum concentrations rapidly, was extensively distributed, and serum concentrations persisted twice as long than in cattle. Whether these characteristics are desirable for the treatment of bacterial respiratory disease in bison species requires confirmation with additional studies designed to assess tulathromycin concentration and persistence in lung tissues in this species. Furthermore, supportive efficacy trials are necessary to confirm whether a dosage regimen recommended for cattle will produce efficacious therapeutic outcomes in bison for infectious respiratory disease.

## **5. PHARMACOKINETICS AND LUNG AND MUSCLE CONCENTRATIONS OF TULATHROMYCIN FOLLOWING SUBCUTANEOUS ADMINISTRATION IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)**

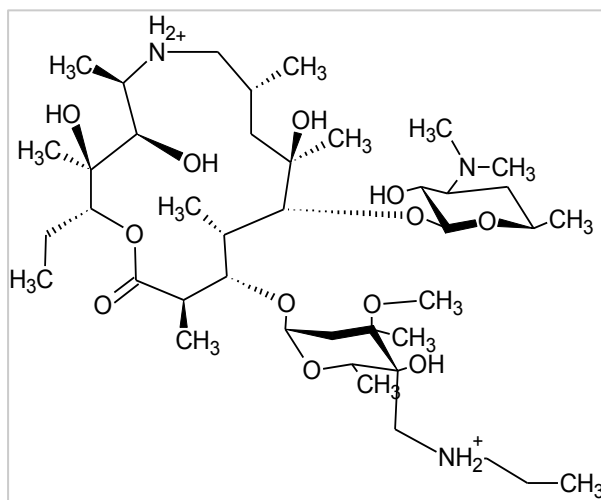
### **5.1 Introduction**

Farming of white-tailed deer (*Odocoileus virginianus*) is a growing industry in North America (NaDeFA, 2013; AWMDA, 2011). Deer are raised commercially for meat and for hunting preserves in North America (Tell et al., 2011). Respiratory infections are a common cause of morbidity and mortality in captive white-tailed deer herds (Tell et al., 2011). Tulathromycin (806.23 g/mol;  $C_{41}H_{79}N_3O_{12}$ ) is a macrolide antibiotic used for the treatment of bacterial respiratory diseases in cattle and swine (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). It is commercially formulated as a long-acting, single-dose injection therapy (APVMA, 2007; Evans, 2005; Gáler et al., 2004; Nowakowski et al., 2004; Benchaoui et al., 2004), thereby avoiding the need for multiple injections to achieve desired therapeutic effects, typical of other macrolide antibiotics, such as erythromycin, tylosin, and spiramycin (Wang et al., 2012; Young et al., 2010; Evans, 2005; Benchaoui et al., 2004; Gáler et al., 2004). Tulathromycin, as a single dose therapy, is desirable in such species as deer where multiple handling is difficult and poses a significant injury risk and stress to such animals. Although tulathromycin is used off-label in deer species, a review of the published literature failed to find reference to the study of this antibiotic in these animals. Pharmacokinetic assessment of tulathromycin in deer is an important component in understanding drug behaviour and efficacy in this species. Evaluation of tissue levels is also important to determine drug persistence and safety for human consumption.

Studies have demonstrated tulathromycin is highly effective for the treatment and prevention of bovine and swine respiratory disease when administered as a single dose (Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Nowakowski et al., 2004). In these species, tulathromycin is rapidly absorbed ( $C_{max}$  <1 hour) and extensively distributed to tissues with accumulation occurring in the lungs (APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004). Elimination half-lives typically range between 3 to 5 days (Drugs.com, 2013; Evans, 2005; Pfizer Inc, 2005; Benchaoui et al., 2004; Gáler et al., 2004). Metabolism is minimal and tulathromycin is eliminated primarily as

unchanged parent drug by biliary excretion (Drugs.com, 2013; APMVA, 2007; Evans, 2005; Nowakowski et al., 2004).

Tulathromycin is a 9:1 ratio mixture of two equilibrating isomers, tulathromycin A (CP-472,295) and B (CP-547,272), in aqueous solution (Drugs.com, 2013; APVMA, 2007; EMEA, 2004). Pharmaceutical analysis often involves the quantitative determination of isomer A (Huang et al., 2012; APVMA, 2007; Scheuch et al., 2007; Evans, 2005; Gáler et al., 2004). We developed a liquid chromatography-mass spectrometry (LC-MS) method for the quantification of tulathromycin in white-tailed deer serum and selected tissues using isomer A (**Figure 5.1**). The purpose of this study was to investigate the pharmacokinetics of tulathromycin in deer following a single 2.5 mg/kg bw subcutaneous injection. We then compared our PK parameter estimates to those in cattle to predict the adequacy of the recommended cattle dose in deer. A small pilot study allowed assessment of lung tissue for inferences of drug efficacy, and muscle tissue to provide a preliminary indication of tissue withdrawal times necessary for safe meat consumption following dosage administration with this drug. This pilot study was also necessary to inform a future, more extensive tissue distribution and withdrawal study of tulathromycin in deer.



**Figure 5.1:** The molecular structure of tulathromycin A (806.23 g/mol; C<sub>41</sub>H<sub>79</sub>N<sub>3</sub>O<sub>12</sub>). The proposed double charged ion, *m/z* 403.9, is shown with ionization most likely localized at the secondary amines and was used for quantitative analysis in deer serum, muscle, and lung.

## 5.2 Materials and Methods

### 5.2.1 Chemicals

Tulathromycin analytical standard was a kind gift from Pfizer Inc. (Groton, CT). The commercially available form of tulathromycin, *Draxxin*, containing 100 mg of tulathromycin/mL (Zoetis Canada, Kirkland, QC), was used to dose deer used in the study. Potassium phosphate dibasic anhydrous buffer,  $K_2HPO_4$ , (Certified, ACS) and ammonium formate were purchased from Fisher Scientific (Ottawa, ON). Formic acid (98% GR ACS) and trifluoroacetic acid (99%) were purchased from VWR (Edmonton, AB). Acetonitrile, methanol, hexane, and ethyl acetate were purchased from Caledon laboratories (Georgetown, Ontario). All chemicals were HPLC grade. Millipore water was prepared by a Milli-Q Advantage water purification system (Fisher Scientific, Ottawa, ON).

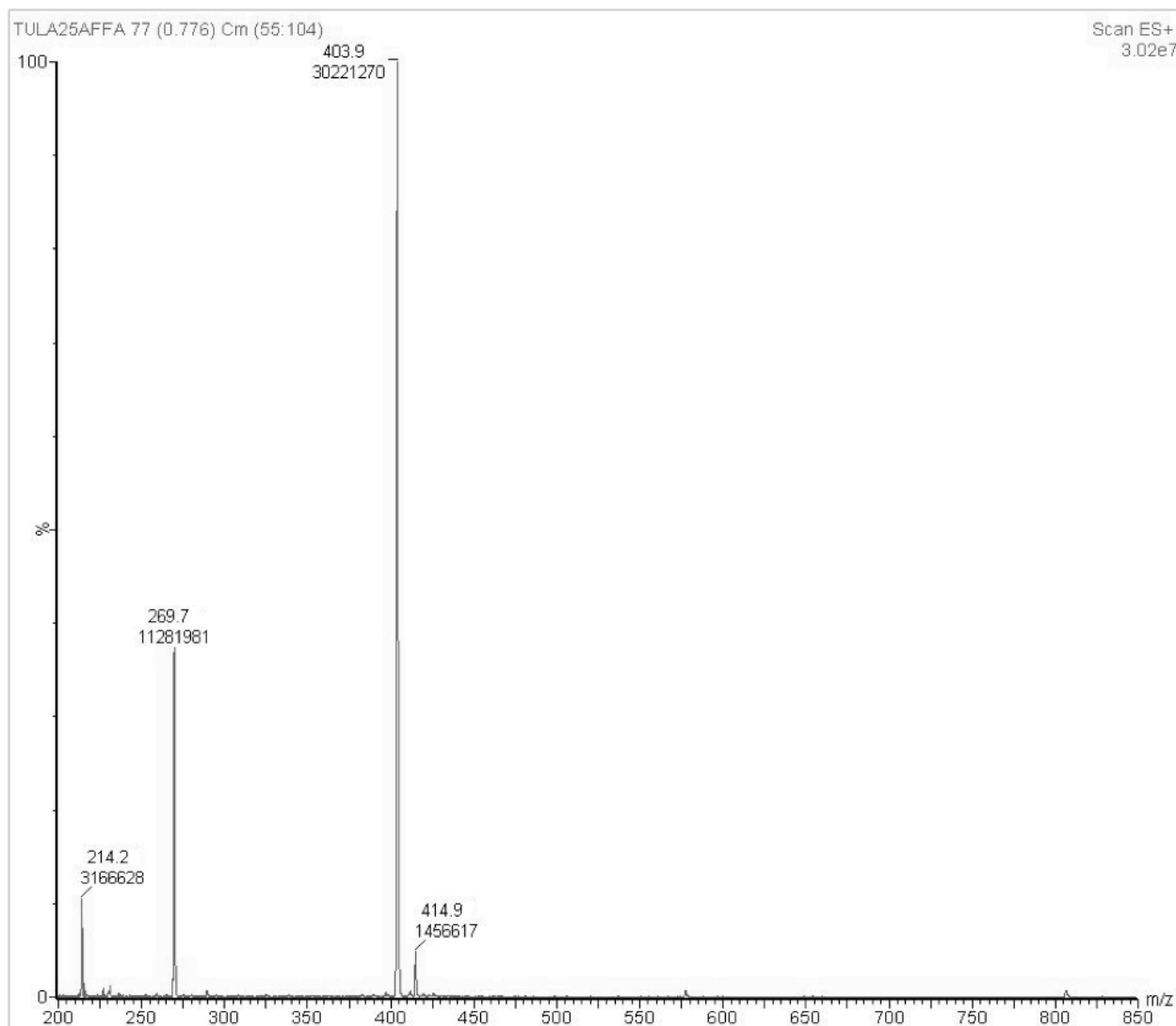
### 5.2.2 Chromatography and Mass Spectrometry

A Waters Alliance 2695 high performance liquid chromatography (HPLC) coupled to a Waters Micromass ZQ single quadrupole mass spectrometer (Waters Corporation, Milford, MA) was used for quantification of tulathromycin. The HPLC system consisted of a pump, degasser, autosampler (set at 10°C), and column heater (set at 55°C). Chromatography was performed by gradient elution using a Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7  $\mu$ m; Agilent Technologies, Ottawa, ON), with a 0.5  $\mu$ m Frit (Canadian Life Science, Edmonton, AB) attached to each end. The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid (A) and 100% methanol (B) delivered at a flow rate of 0.35 mL/min. The gradient was as follows: 95% A for 5 min, 5% A until 10.8 min, and a return to initial mobile phase conditions, 95% A, at 11 min. Total run time was 17 min, with the analyte eluting at 4.6 min.

Electrospray ionization (ESI) was used in positive ionization mode. Compound optimization was performed by infusion using a model 11 Plus syringe pump (Waters Corporation, Milford, MA). Ions were monitored in selected ion monitoring (SIM) mode, with the double charge ion  $[M+2H]^{+2}$  at  $m/z$  403.9 used for quantification (**Figure 5.1**). The positive ion ESI-MS spectrum of tulathromycin is shown in **Figure 5.2**. Optimal instrument parameters are presented in **Table 5.1**.



MassLynx version 4.1 (Waters Corporation, Milford, MA) was used to acquire and quantify the data, while Microsoft Office Excel 2007 (Microsoft Corporation) and Minitab version 12.1 (Minitab Inc., 1998) were used for statistical evaluation.



**Figure 5.2:** Positive ion electrospray mass spectra of tulathromycin obtained on a Waters Micromass ZQ mass spectrometer. The double charge ion was observed at  $m/z$  403.9.

**Table 5.1:** Optimal instrument parameters for the determination of tulathromycin in deer serum using positive ion electrospray ionization. Selected ion monitoring (SIM) mode was used using a Waters Micromass ZQ mass spectrometer instrument.

Variable	Value
Cone gas	50 L/hr
Desolvation gas	1100 L/hr
Capillary voltage	1.70 kV
Cone voltage	25 V
Source temp.	120°C
Desolvation temp.	450°C

### 5.2.3 Pharmacokinetic Study

Ten white-tailed deer (*Odocoileus virginianus*, female, mixed age adults) were used for pharmacokinetic study of tulathromycin. The animals were housed at the Specialized Livestock Research Facility, University of Saskatchewan. Animals were fed on pasture forage during the summer months, and baled forage with mineral supplements in the winter months. Fresh water was available *ad libitum*. Routine preventative health care was provided consisting of annual application of anthelmintic (*Ivomec*, Merial Canada Inc., Baie-D'Urfe, QC) and a multivalent Clostridial vaccine (*Covexin 8*, Schering-Plough Canada Inc., Kirkland, QC). Animals had no known history of macrolide exposure. The study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Animals were given a single 2.5 mg/kg bw subcutaneous injection of *Draxxin* (Zoetis Canada, Kirkland, QC) in the neck; the therapeutic dose and route of administration approved for cattle. Blood samples were collected in 10 mL serum (red top) vacutainer tubes by jugular venipuncture at 1, 2, 4, 12 hours, and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, 19, 21, 23, and 25 days post-injection. A blood sample was taken from each animal prior to dosing for use as a blank control. Animals were observed daily for general health and clinical observations were made prior to injection and at collection times post-dose. Serum was separated from blood samples by centrifugation (Eppendorf 5804 R Centrifuge) at 1500 x g for 10 min, transferred to microcentrifuge tubes, and stored at -80°C until analysis.

#### **5.2.4 Tissue Withdrawal Pilot Study**

Thirteen deer were given the same dose of tulathromycin used in the pharmacokinetic study (2.5 mg/kg bw subcutaneous injection in the neck). To serve as controls, three animals did not receive an injection. Two to three deer were slaughtered per time point at 1, 2, 6, 7, and 8 weeks post-injection. Animals were suitably restrained in a species-specific squeeze chute and killed with a captive bolt gun designed to humanely euthanize small ruminants. Lung and muscle tissues were collected immediately following euthanasia, as were kidney, liver, adipose, and heart. At least two samples were collected per tissue. Tissues were stored at -80°C pending analysis. All samples were analyzed in triplicate (i.e. 2 lung samples, each analyzed in triplicate gives an n of 6). The study was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

#### **5.2.5 Serum Assay Procedure**

Blank (free of analyte) blood samples were collected from at least ten white-tailed deer for use in the serum assay and validation. Blood samples were centrifuged (Eppendorf 5804 R Centrifuge) and serum transferred into polypropylene centrifuge tubes. Serum was pooled before use in analysis and stored at -20°C.

A 1.0 mg/mL (1000 µg/mL) stock solution of tulathromycin was prepared by dissolving the tulathromycin standard in 10 mL of acetonitrile. Working standard solutions of 10 µg/mL and 200 ng/mL were prepared by diluting 100 µL of the 1000 µg/mL stock solution, or 200 µL of the 10 µg/mL working standard solution, into 10 mL acetonitrile, respectively. Stock standard was prepared quarterly and stored at -20°C, while working standards were made fresh before each analysis. Drug-free serum samples (1.0 mL) were spiked with working standard solutions of tulathromycin to prepare the calibration curve and quality control samples prior to extraction.

To 1.0 mL of serum, 100 µL of acetonitrile and 20 µL of trifluoroacetic acid were added to each sample. After mixing and centrifuging (Refrigerated Microcentrifuge Z 216 MK, Hermle Labor Technik) at 21380 x g for 15 min at 5°C, 0.5 mL was transferred to a new 1.5 mL centrifuge tube (MCT Graduated Natural, Fischer Scientific) and diluted with 0.5 mL of 50 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.8). Samples were centrifuged a second time to remove particulates and

subsequently loaded onto preconditioned solid phase extraction cartridges (Bond Elut-CBA 50 mg bedmass, 1 mL tube volume; Agilent Technologies, Ottawa, ON). Cartridges were preconditioned twice with 1.0 mL acetonitrile and twice with 1.0 mL of the 50 mM  $\text{K}_2\text{HPO}_4$  buffer solution. The samples were washed thoroughly with 1.0 mL  $\text{K}_2\text{HPO}_4$  (x2), 1.0 mL Millipore Water (x4), 1.0 mL acetonitrile (x4), and 1.0 mL ethyl acetate containing 2% formic acid (x2). The cartridges were dried under vacuum for 7 min before eluting 3 times with 1.0 mL methanol containing 2% formic acid. Eluates were evaporated to dryness under nitrogen in a 40°C water bath and dissolved in 200  $\mu\text{L}$  methanol and 300  $\mu\text{L}$  of 1% formic acid, vortex mixed, and sonicated for 15 min before being transferred to an LC filter vial (Whatman Mini-Uniprep Syringeless Filter, PTFE, 0.2  $\mu\text{m}$ ; VWR, Edmonton, AB). A 5  $\mu\text{L}$  sample was injected for LC-MS analysis.

#### **5.2.6 Tissue Homogenate Assay Procedure**

Blank (free of analyte) white-tailed deer tissues were obtained from the three untreated animals and the tissues were used for preparation of calibration standards and quality control samples. Lung and muscle tissues were homogenized using a food processor to obtain a uniformly blended homogenate. Samples were placed into Whirl-Pak bags and frozen until ready for analysis. Each unknown sample was analyzed in triplicate. Tissue samples ( $2.0 \text{ g} \pm 0.05$ ) were mixed with 5.0 mL acetonitrile and 5.0 mL of 50 mM  $\text{K}_2\text{HPO}_4$  (pH 6.8) in a 50 mL centrifuge tube (Falcon, Fisher Scientific, Ottawa, ON). Samples were vortex mixed for approximately 1 min, shaken (Eberbach Corporation, VWR, Edmonton, AB) for 15 min, and centrifuged (Mandel Sovall RC 5C Plus, Guelph, ON) at  $3917 \times g$  for 10 min. The supernatant was transferred to a new 50 mL centrifuge tube and an additional 2.5 mL of acetonitrile and 2.5 mL of 50 mM  $\text{K}_2\text{HPO}_4$  were added to the tissue pellet. Samples were vortex mixed, shaken, and centrifuged a second time and the resulting supernatant was transferred to the same tube containing the supernatant from the first extraction. Supernatants were centrifuged ( $3917 \times g$ ) to remove any particulates before clean up procedures by solid phase extraction. Cartridges (Bond Elut –CBA, 200 mg, 3 mL, Agilent Technologies, Ottawa, ON) were preconditioned with 3.0 mL acetonitrile and 3.0 mL of buffer (50 mM  $\text{K}_2\text{HPO}_4$ , pH 6.8). Glass wool was added to each cartridge and the sample was loaded. Cartridges were washed twice with 3.0 mL buffer, removing the glass wool

after the first wash, followed by 3.0 mL Millipore water (x3), 3.0 mL ethyl acetate, 3.0 mL hexane, and 3.0 mL ethyl acetate containing 2% formic acid. Cartridges were dried for 15 min before eluting 3 times with 1.0 mL methanol containing 2% formic into 15 mL centrifuge tubes. The total volume was adjusted to 10.0 mL with Millipore water. Each tube was mixed and a 500  $\mu$ L subsample was transferred to an LC filter vial (Whatman Mini-Uniprep Syringeless Filter, PTFE, 0.2  $\mu$ m; VWR, Edmonton, AB), of which 5  $\mu$ L was injected for LC-MS analysis.

All spiked samples (calibration or QC) were prepared by adding appropriate aliquots of a working standard to 2.0  $\pm$ 0.05 g of homogenized tissue, letting the sample settle for 15 min, then extracting and cleaning up the sample as indicated above. From a 1.0 mg/mL (1000  $\mu$ g/mL) stock standard solution, working standards of 20  $\mu$ g/mL and 50  $\mu$ g/mL were prepared for muscle and lung tissue analysis, respectively.

### 5.2.7 Pharmacokinetic Data Analysis

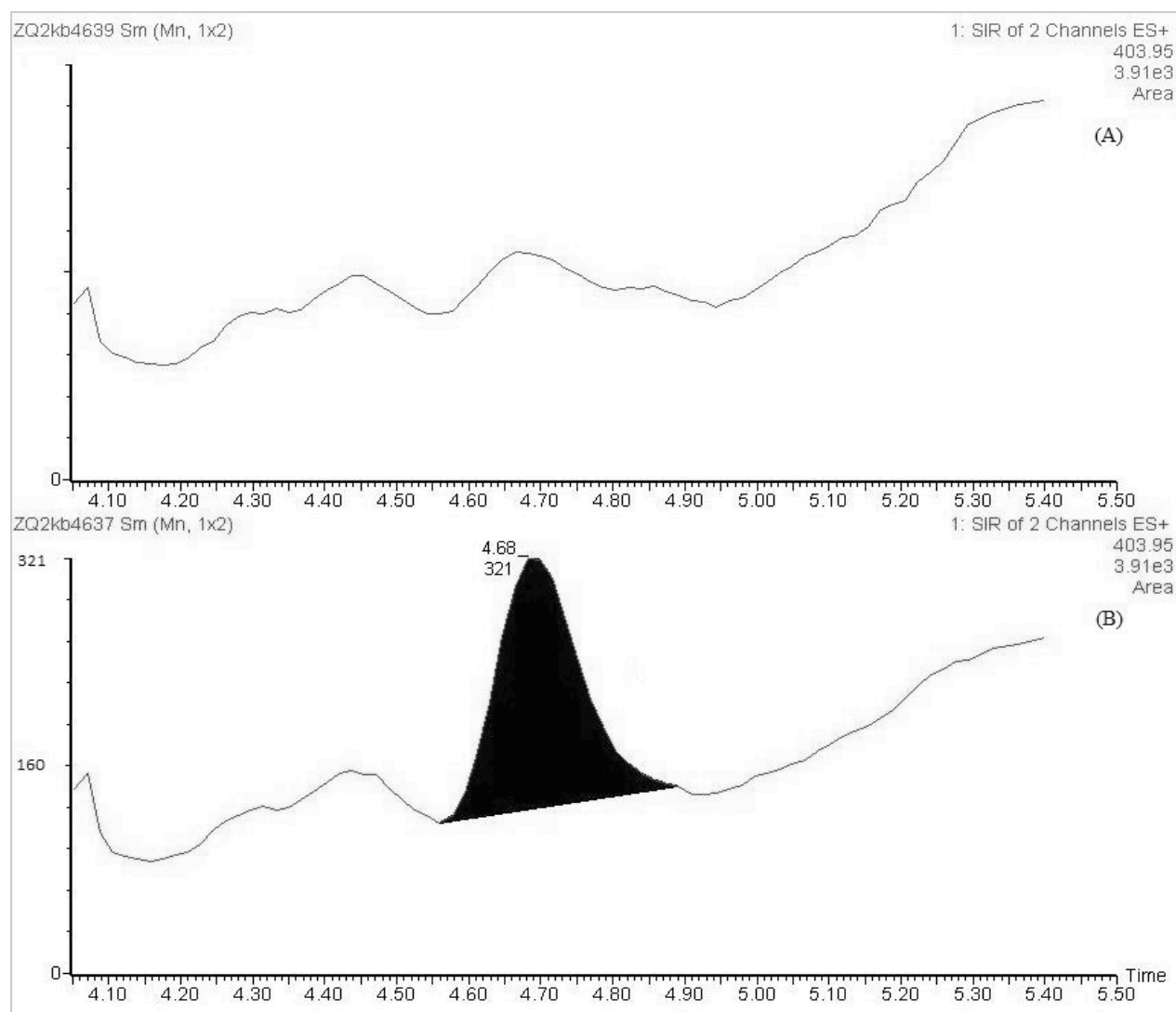
A non-compartmental approach was used to analyze the serum concentration versus time data. Pharmacokinetic (PK) parameters were estimated for each individual deer serum concentration-time curve using GraphPad Prism software (Version 5.0, San Diego, California). The PK parameters estimated from each individual animal included the log-linear terminal rate constant ( $k$ ), area under the serum concentration-time curve extrapolated to infinity ( $AUC_{0-inf}$ ), area under the first moment serum concentration-time curve extrapolated to infinity ( $AUMC_{0-inf}$ ), half life ( $t_{1/2}$ ), mean residence time (MRT), maximum plasma concentration ( $C_{max}$ ) and the time to  $C_{max}$  ( $t_{max}$ ).  $C_{max}$  and  $t_{max}$  were taken visually from the serum concentration-time curves. The  $AUC_{0-inf}$  and  $AUMC_{0-inf}$  values were calculated using the trapezoidal rule-extrapolation method (assessed up to the last sampling time and then extrapolated to infinity).  $k$  was calculated from the slope of the terminal portion of the natural logarithm concentration versus time plot, and half-life was calculated as the ratio of 0.693 to  $k$ . The apparent clearance ( $Cl/F$ ) and apparent volume of distribution ( $V_d/F$ ) were also calculated, as  $dose/AUC_{0-inf}$  and  $dose/AUC_{0-inf} * k$ , respectively. The MRT was calculated from the ratio of  $AUMC_{0-inf}$  to  $AUC_{0-inf}$ . PK parameter estimates were calculated for each individual animal and reported as the mean and standard deviation of all individual animals.

## 5.3 Results

### 5.3.1 Serum Assay Validation Summary

Selectivity of tulathromycin in deer serum was tested by comparing blank deer serum to blank deer serum spiked with tulathromycin in replicates of five. **Figure 5.3** shows a representative chromatogram of a blank matrix fortified sample and a 0.6 ng/mL tulathromycin spiked sample in deer serum. Both chromatograms were obtained after extraction. LC-MS detection of the double charge ion at  $m/z$  403.9 was specific to tulathromycin. The calibration curve was linear from 0.6 ng/mL to 50 ng/mL with a coefficient of determination ( $r^2$ ) >0.99. The limit of detection (LOD) was statistically calculated as 0.2 ng/mL, and the limit of quantitation (LOQ) as 0.6 ng/mL. A weighting factor of  $1/x$  was applied to the calibration curve to compensate for the heteroscedasticity of the regression data.

Intra- and inter-day accuracy and precision were determined by analysis of six replicates of quality control (QC) samples at three different concentrations (1.5 ng/mL, 15 ng/mL, and 40 ng/mL) on two different days. QCs were prepared using blank sample spiked with standard solutions at low (LQC), middle (MQC), and high (HQC) concentrations. Values for the QCs fell within the acceptable criteria ( $\pm 15\%$ ). Dilution integrity was validated by extracting known amounts of spiked samples followed by 2-fold, 5-fold, and 10-fold dilution with blank extract matrix in replicates of at least two; accuracy and precision were <15%. Recovery of tulathromycin was assessed by comparing calibration samples spiked with tulathromycin before extraction (matrix fortified) with those spiked after extraction (matrix matched). Recovery of tulathromycin in deer serum was 32%. Matrix enhancement was also noted, where analytical results for extracted samples (matrix fortified) were compared to chemical standards that represent 100% recovery. In other words, deer serum increased the signal for the analyte resulting in ion enhancement.



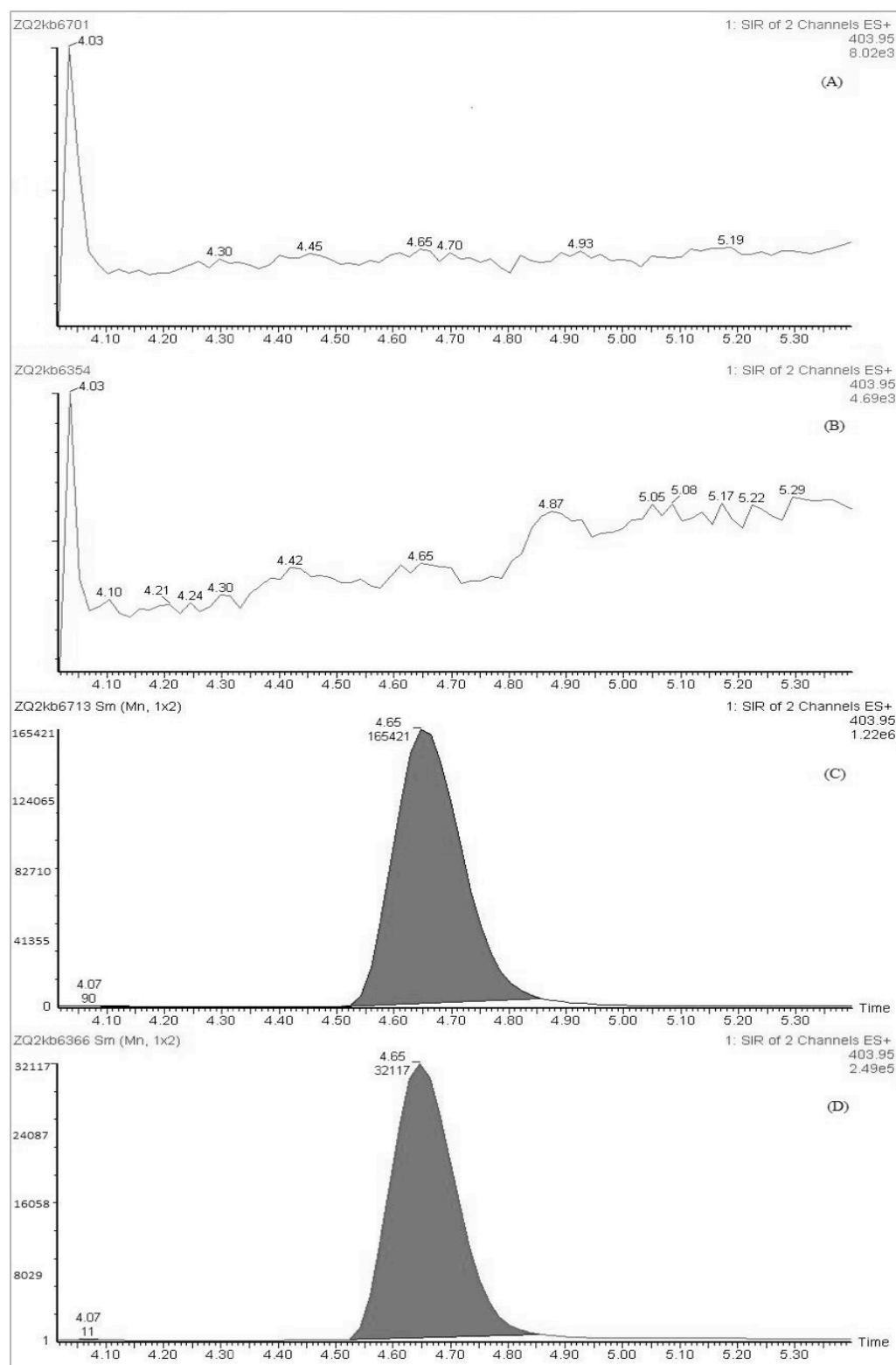
**Figure 5.3:** Representative chromatogram of an extract of a blank deer serum sample (A) and a blank deer serum sample fortified with 0.6 ng/mL tulathromycin (B).

### 5.3.2 Tissue Assay Validation Summary

Representative chromatograms of blank (drug-free) and fortified tulathromycin lung and muscle tissues of white-tailed deer are shown in **Figure 5.4**. Calibration curve ranges were selected as 500 ng/g to 5000 ng/g and 100 ng/g to 2500 ng/g to fall within the expected values for tulathromycin concentrations in white-tailed deer lung and muscle tissues, respectively. As such, LOQ and LOD were not determined. Calibration curves were linear ( $r^2 > 0.99$ ) and a weight of  $1/x$  was applied to each calibration curve. Intra- and inter-day accuracy and precision values were  $\pm 15\%$  for both lung and muscle tissues and are reported in **Tables 5.2** and **5.3**. Recovery of

tulathromycin in white-tailed deer lung was 68%, while the recovery in muscle was >90%. Matrix signal enhancement was noted for lung tissues, while matrix signal suppression was observed for muscle tissues under the ESI mass spectrometry conditions used for the analysis.





**Figure 5.4:** Representative tulathromycin chromatograms in white-tailed deer tissues: blank deer lung tissue (A), blank deer muscle tissue (B), deer lung tissue spiked at 500 ng/g (C), and deer muscle tissue spiked at 100 ng/g (D).

**Table 5.2:** Intra-day accuracy and precision values for tulathromycin in white-tailed deer muscle and lung.

QC Sample*	Muscle		Lung	
	Precision (%) (n=6)	Accuracy (%) (n=6)	Precision (%) (n=6)	Accuracy (%) (n=6)
LQC				
Day 1	1.9	-0.4	1.7	3.2
Day 2	2.0	-1.3	2.0	3.0
Day 3	6.7	1.6	1.8	1.3
MQC				
Day 1	2.4	2.7	1.7	-3.3
Day 2	1.9	-0.5	1.9	-1.4
Day 3	2.6	2.3	2.2	-0.9
HQC				
Day 1	2.2	3.0	1.8	-1.3
Day 2	1.0	-1.9	1.4	0.9
Day 3	2.8	1.8	1.8	-0.7

\*Concentrations used for the QCs in deer muscle were: 300 ng/g (LQC), 700 ng/g (MQC), 2200 ng/g (HQC), and in deer lung: 600 ng/g (LQC), 2000 ng/g (MQC), 4700 ng/g (HQC).

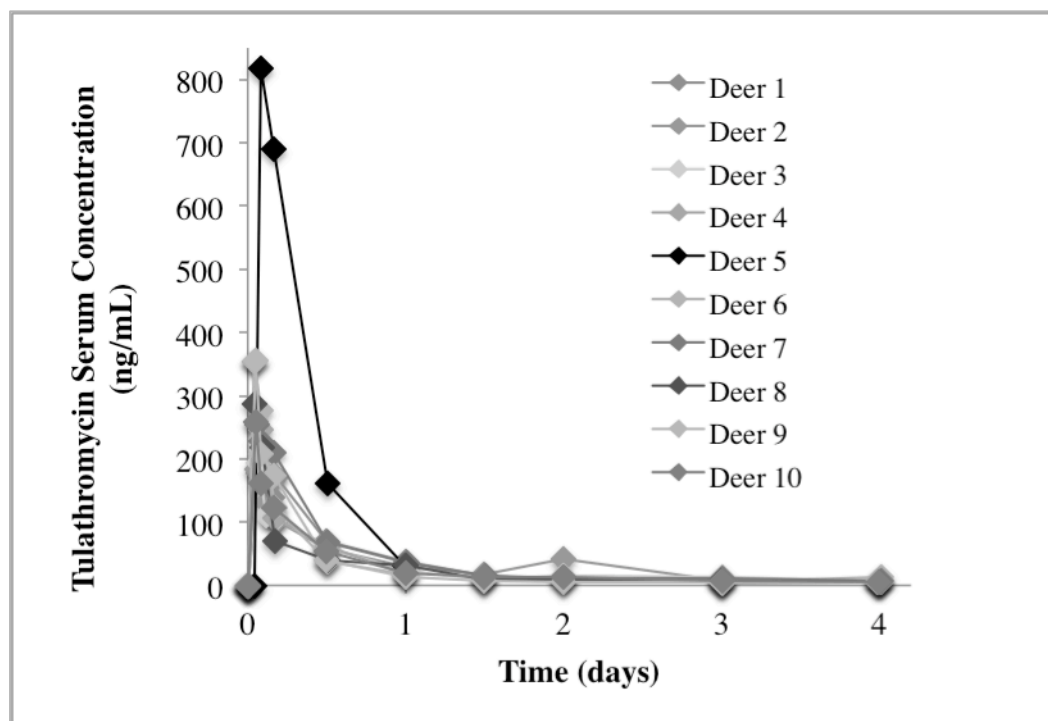
**Table 5.3:** Inter-day accuracy and precision values for tulathromycin in white-tailed deer muscle and lung, calculated over a three-day period.

Tissue	QC Concentration	Precision (%) (n=18)	Accuracy (%) (n=18)
Muscle	300 ng/g	3.7	-0.1
Muscle	700 ng/g	2.6	1.7
Muscle	2200 ng/g	2.9	1.3
Lung	600 ng/g	1.9	2.6
Lung	2000 ng/g	2.1	-2.1
Lung	4700 ng/g	1.8	-0.5

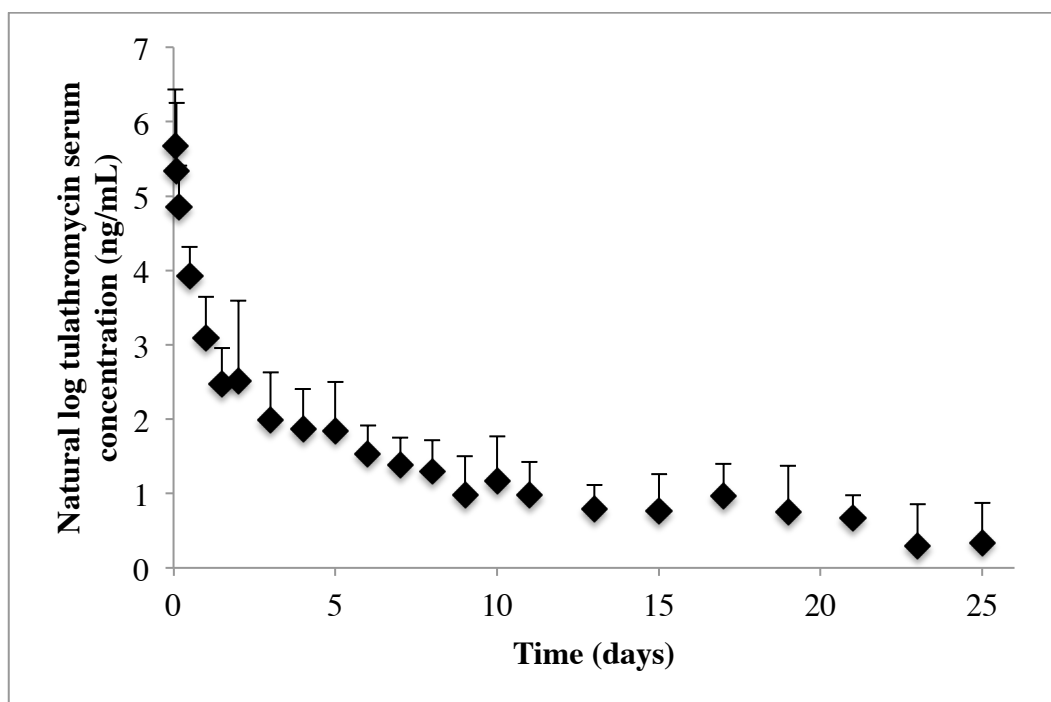
### 5.3.3 Tulathromycin Pharmacokinetics in Deer

Serum concentration-time profiles of tulathromycin following a 2.5 mg/kg bw subcutaneous injection in the ten deer are shown in **Figures 5.5 and 5.6**. These figures demonstrate extensive interindividual variation in tulathromycin concentration-time profiles and two-compartment kinetics with a prolonged distribution phase. One animal was removed from the study after day 15 due to injury; however, sufficient data points to assess the terminal phase

allowed PK parameter estimation for this animal and it was included in the reported mean values. Mean and standard deviation of each PK parameter estimate are reported in **Table 5.4**. Maximal serum concentrations ( $C_{\max}$ ) ranged from 177 to 817 ng/mL, with a mean  $C_{\max}$  of 359 ng/mL. The time to reach maximal serum concentration ( $t_{\max}$ ) was 1.3 hours in deer.  $AUC_{0-\infty}$  values were between 3421 and 5687 ng\*h/mL, with a mean  $AUC_{0-\infty}$  of 4883 ng\*h/mL. Apparent volume of distribution ( $V_d/F$ ) following subcutaneous injection ranged between 121 and 288 L/kg. Half-lives ranged between 151 and 454 hours (6 to 18 days). The mean half-life of tulathromycin was 281 hours (11.7 days), while the mean residence time was 217 hours.



**Figure 5.5:** Tulathromycin serum concentrations versus time of ten individual female deer following a 2.5 mg/kg bw subcutaneous injection of *Draxxin*. The data has been truncated, showing data up to 4 days to give a better indication of variability and AUC.



**Figure 5.6:** Natural-logarithmic plot of mean and standard deviation tulathromycin concentrations over time following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* to ten female deer.

**Table 5.4:** Mean and standard deviation (SD) of each tulathromycin PK parameter following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* to ten female deer.

PK parameter	Mean (n=10)	SD
k (h <sup>-1</sup> )	0.0027	0.0009
AUC <sub>0-inf</sub> (ng*h/mL)	4883	733
C <sub>max</sub> (ng/mL)	359	224
t <sub>max</sub> (h)	1.3	0.2
Cl/F (L/h/kg)	0.5	0.1
V <sub>d</sub> /F (L/kg)	208	52
t <sub>1/2</sub> (h)	281	86
AUMC <sub>0-inf</sub> (µg*h <sup>2</sup> /mL	1087	466
MRT (h)	217	77

### 5.3.4 Muscle and Lung Tissue

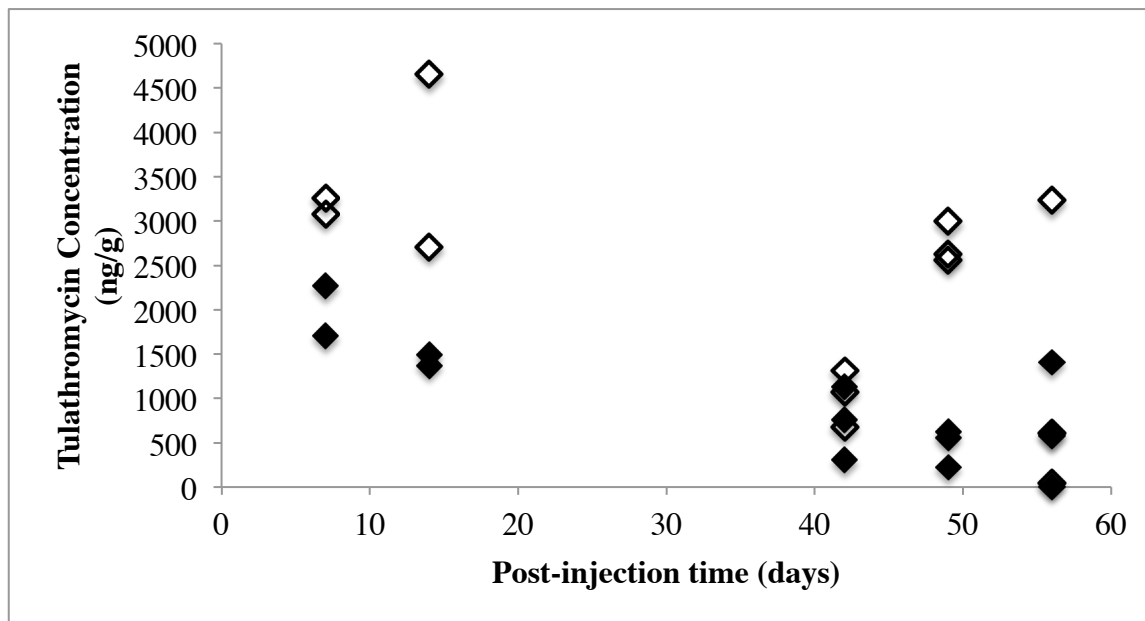
Mean and standard deviation of tulathromycin muscle and lung concentrations in the thirteen white-tailed deer are reported in **Table 5.5**. Muscle and lung tissue depletion profiles are shown in **Figure 5.7**. Animals exhibited extensive interindividual variation following a 2.5 mg/kg bw subcutaneous injection of *Draxxin*, as high variability between animals over time is evident. Deer had high quantifiable levels of tulathromycin in both lung and muscle tissues over the entire time course of the study (56 days). Tulathromycin concentrations were greatest in the first 1 to 2 weeks (first and second sampling time points). The maximal tulathromycin concentration in lung homogenate from a single animal was 4657 ng/g (14 days), and the minimum was 39.4 ng/g (56 days). The maximum concentration in muscle homogenate from a single animal was 2264 ng/g (7 days), and the minimum was 9.1 ng/g (56 days). In general, tulathromycin concentrations were higher in lung tissues relative to muscle tissues. Detectable levels were still present 56 days after treatment in white-tailed deer. This preliminary investigation of tulathromycin in deer tissues identified the need for earlier sampling time points (24 hours) to help with drug efficacy predictions, as well as later time points (>56 days), which would ensure when the drug is completely eliminated.

**Table 5.5:** Mean and standard deviation (SD) of representative tulathromycin muscle and lung tissue concentrations in thirteen female white-tailed deer following a 2.5 mg/kg bw subcutaneous injection of *Draxxin* over time.

Animal ID	Time after treatment (days)	Muscle		Lung	
		Mean (ng/g) n=6	SD	Mean (ng/g) n=6	SD
40T	7	1702.7	97.3	3080.3	329.2
69N	7	2263.5	53.7	3260.0	58.0
21N	14	1367.1	455.9	2702.3	58.1
51T	14	1495.4	25.3	4657.0	755.3
66S	42	302.1	8.5	1072.3	92.8
198P	42	1130.4	57.5	1310.1	38.2
16T	42	758.9	20.9	680.0	14.1
85T	49	555.8	136.9	2992.6	187.0
71U	49	221.4	8.5	2564.0	196.9
38N	49	623.5	96.1	2727.4	135.3

92R	56	9.1*	0.5	39.4*	5.4
51N	56	578.3	13.4	602.4	28.5
32M	56	1407.8	92.2	3231.8	97.5

\*Concentration was outside the standard curve range. The standard curve was diluted to fall within the appropriate range and the sample re-ran to get a more accurate value.



**Figure 5.7:** Lung (◊) and muscle (◆) tulathromycin concentrations over time in thirteen deer following a 2.5 mg/kg bw subcutaneous injection of *Draxxin*.

## 5.4 Discussion

The pharmacokinetics of tulathromycin in white-tailed deer following a dosage regimen recommended for cattle are reported here for the first time. No adverse effects occurred in the present studies for deer administered 2.5 mg/kg bw of the commercial form of tulathromycin. Tulathromycin reached maximal serum concentrations within 1 hour after subcutaneous injection, consistent with values reported in cattle (Gáler et al., 2004; Nowakowski et al., 2004). The mean  $C_{\max}$  (359 ng/mL) is comparable to those reported in cattle at 300 ng/mL (Gáler et al., 2004) and 500 ng/mL (Nowakowski et al., 2004), while the mean deer  $AUC_{0-\infty}$  (4883 ng\*hr/mL) was lower compared to cattle reported at 18700 ng\*hr/mL (Nowakowski et al., 2004). The half-life of

tulathromycin in deer serum (281 hours) was much longer compared to cattle at 90 to 110 hours (Gáler et al., 2004; Nowakowski et al., 2004). Tulathromycin pharmacokinetic characteristics reported in other species, including swine (Huang et al., 2012; Benchaoui et al., 2004), goats (Young et al., 2010), and foals (Scheuch et al., 2007), also present similar values to cattle.

The bioavailable dose is an important determinant of both AUC and  $C_{\max}$  values. As compared to cattle, tulathromycin  $C_{\max}$  values were similar, suggesting bioavailability following subcutaneous injection is comparable in deer as it is in cattle and other species. Systemic clearance also determines AUC and  $C_{\max}$  values, and more rapid elimination kinetics in deer may explain the lower AUC value in deer relative to other species. Half-life depends on both systemic clearance and volume of distribution. Serum half-life is likely elevated due to extensive distribution into tissues.

Following subcutaneous administration, tulathromycin was extensively distributed to the tissues (lung and muscle), which was expected for this drug. Peak concentration was highest in lung tissues (lung  $C_{\max}$  = 4657 ng/g), followed by muscle (muscle  $C_{\max}$  = 2263 ng/g). The maximal tissue concentration in lung and muscle was reached within 7 to 14 days (first and second sampling time points) after injection. Our first sampling time point was 7 days, however maximal tissue concentration may have been reached sooner. Studies in cattle and swine found lung homogenates had highest concentration 24 hours after dosing (Evans, 2005; Kilgore, et al., 2005; Benchaoui et al., 2004; Nowakowski, 2004). Drug concentrations in the tissues analyzed were markedly higher than those in plasma. Macrolides in general are lipophilic and have a low degree of ionization, which allows for extensive drug penetration and a large volume of distribution (Benchaoui et al., 2004; Nowakowski et al., 2004; Zhanel et al., 2001). Triamilides (tulathromycin) are basic compounds and contain 3 amines within their structure with pKa values of 8.5, 9.3, and 9.8 (Reeves, 2012). These properties allow tulathromycin to achieve high tissue penetration (Benchaoui et al., 2004; Zhanel et al., 2001). The apparent volume of distribution of tulathromycin was extensive (208 L/kg) and the high lung and muscle drug concentrations are supportive of this PK feature. Analysis of tulathromycin in other tissues (kidney, liver, adipose) was not assessed in the present study; however, tulathromycin also reaches high concentration in other tissues, such as liver and kidney (APVMA, 2007; EMEA, 2004). Although there was not sufficient lung tissue data (data points) to calculate lung half-life, other species demonstrated a slow decline in lung drug concentration, with a lung half-life of 8 days in cattle (Nowakowski et

al., 2004) and 6 days in swine (Benchouai et al., 2004). In the present study, drug concentrations in lung were still detected 56 days after treatment, demonstrating a slow elimination of tulathromycin from the lung. This characteristic suggests tulathromycin can be administered as a single injection therapy and results in prolonged potential exposure of tulathromycin to the bacterial pathogens associated with respiratory disease of deer.

In Canada, the maximum residue limit (MRL) of tulathromycin drug residues in cattle is 1.0 µg/g in muscle tissue (Health Canada-2, 2013). The withdrawal time, which ensures when the tissues have depleted to safe levels for human consumption is 44 days for tulathromycin in cattle (Drugs. com, 2013). [Note that these values are established based on CP-60,300, the established marker residue for tulathromycin.] There is no MRL established for deer because this drug is not approved for use in deer. In cases where no MRL has been established for a specific veterinary drug in a species, no detectable residues are permitted in tissues when a drug is used off-label. In the present study, concentrations up to 1.4 µg/g were measured in muscle tissue 56 days after treatment. Based on our data, the use of tulathromycin in deer requires a much longer withdrawal interval, perhaps greater than 56 day, in comparison to the times established for cattle (44 days). Further studies are necessary to determine an accurate withdrawal interval for tulathromycin in deer species.

Although the pharmacodynamics (PD) of tulathromycin in deer was not part of the present study, both PK and PD properties are important in determining antimicrobial efficacy (Evans, 2005; Zhanel et al., 2001). Older PD studies using macrolides suggest time above the minimum inhibitory concentration ( $T > MIC$ ) corresponds with clinical efficacy (Zhanel et al., 2001; Nightingale, 1997). Alternatively, newer macrolides (ex: azithromycin), which have prolonged persistence, associate AUC/MIC or total drug exposure to the pathogen to be more correlated with efficacy (Evans, 2005; Kilgore et al., 2005; Zhanel et al., 2001). This may be because newer macrolides achieve prolonged half-lives in the target tissue allowing for persistent exposure of pathogens to the drug (Kilgore et al., 2005; Nowakowski et al., 2004).

The MIC<sub>90</sub> (lowest concentration inhibiting 90% of isolates) values for tulathromycin against common respiratory pathogens in cattle are: *M. haemolytica* 2 µg/mL, *P. multocida* 1 µg/mL, *H. somni* 4 µg/mL and *M. bovis* 1 µg/mL (Evans, 2005; Kilgore, 2005). While *M. bovis* has been documented in deer species (Dyer et al., 2004), the MIC has not. Assuming deer have the same MIC values for this pathogen found in cattle (1 µg /mL), tulathromycin has the potential



to be an effective treatment for respiratory disease in this species because concentrations in lung tissue in the present study were above the MICs for a prolonged period. The  $T > MIC$  for tulathromycin against *M. bovis* would be approximately 14 days in lung tissue. At 14 days after treatment, lung concentrations were 2702 to 4657 ng/g (2.7 to 4.6 µg /mL) and at the next time point, 42 days after treatment, lung concentrations were 680 to 1310 ng/g (0.6 to 1.3 µg /mL). Furthermore, the AUC:MIC ratio suggest the 2.5 mg/kg bw subcutaneous dose might exhibit adequate clinical efficacy in deer. However, *Arcanobacterium pyogenes* has been isolated from lungs of white-tailed deer with pneumonia (Tell et al., 2011). The MIC for tulathromycin against this pathogen was determined to be  $\leq 16$  µg /mL (Tell et al., 2011). The  $C_{max}$  in lung at 14 days (4.6 µg /mL) is well below the effective concentration for this respiratory pathogen in deer, as is the AUC:MIC ratio. Determining the  $C_{max}$  in lung tissues at earlier time points in deer (24 hours) will help in further assessing the pharmacodynamics of tulathromycin in this species, as will studies evaluating  $MIC_{90}$  for *M. bovis* in deer.

The tulathromycin concentration in serum (359 ng/mL or 0.3 µg /mL) was below the MIC values for the above reported pathogens. It is generally accepted that serum or plasma concentrations of macrolides are poor predictors of efficacy and tissue concentrations at the site of infection are more helpful in understanding the PD of this class of compound (Evans, 2005; Benchaoui et al., 2004; Nowakowski et al., 2004; Nightingale, 1997). In the present study, lung homogenates were analyzed to assess pulmonary exposure and drug persistence. These lung homogenates represent a mixture of intracellular and extracellular drug concentrations, making it difficult to interpret results for this antibacterial that mainly target extracellular pathogens, such as *M. bovis*. Even so, lung tissue concentrations provide some understanding of the PK/PD relationship of tulathromycin that cannot be explained by serum concentration alone (Benchaoui et al., 2004). Total lung tissue concentrations correlate better with the extent of exposure at the site of infection and with *in vivo* antibacterial activity, than plasma or serum concentrations (Benchaoui et al., 2004). The mechanism of activity against extracellular pathogens, such as *M. bovis*, has been documented for modern macrolides, including tulathromycin (Evans, 2005; Kilgore et al., 2005; Zhanel et al., 2001). Data suggest tulathromycin concentrations in infected lungs are due to a tissue-targeting occurrence (Evans, 2005). In other words, tulathromycin is taken up by immune cells that travel to the lung tissue in response to inflammation caused by the bacterial infection (Evans, 2005; Kilgore et al., 2005; Zhanel et al., 2001). Tulathromycin

accumulates in phagocytes, specifically blood polymorphnuclear leukocytes or neutrophils (PMNs) and alveolar macrophages (Evans, 2005; Kilgore et al., 2005; Zhanel et al., 2001). Greater ionization of tulathromycin (tribasic) may lead to trapping of the protonated drug in the neutrophil granules (Zhanel et al., 2001). This accumulation is followed by a slow release from these cell types into the extracellular environment where it would be available to attack respiratory pathogens (Evans, 2005; Zhanel et al., 2001). Drug efflux from the phagocytes allows for tulathromycin concentrations to remain higher at the site of infection than in lung homogenates (Kilgore et al., 2005). Further work is needed to evaluate drug exposure in the lung extracellular environment, which would help determine the best PK/PD index for predicting *in vivo* efficacy for tulathromycin in deer.

## 5.5 Conclusion

An LC-MS method was developed and validated for tulathromycin in white-tailed deer serum and lung and muscle tissue following international criteria for bioanalytical method validation. In deer, a single 2.5 mg/kg bw subcutaneous injection of tulathromycin achieved maximum serum concentrations rapidly and was extensively distributed to lung and muscle tissues where high drug concentrations persisted for an extended period of time. These characteristics are desirable for the treatment of bacterial respiratory disease in deer species. Based on similar  $C_{\max}$  values in deer as those reported in cattle and high lung tissue concentrations in deer, we predict the 2.5 mg/kg dosage regimen recommended for cattle is effective for similar infections in deer. Further, tissue distribution and depletion studies are necessary to investigate the persistence of tulathromycin in deer, which will provide important information regarding appropriate withdrawal times in this species.

## 6. SUMMARY DISCUSSION, CONCLUSIONS, AND FUTURE WORK

Tulathromycin is approved for use in bovine and swine respiratory disease and is formulated as a long-acting, single-dose injection therapy (Drugs.com, 2013; APVMA, 2007; Evans, 2005; Benchaoui et al., 2004; EMEA, 2004; Gáler et al., 2004; Nowakowski et al., 2004), making it of particular interest for use in bison and deer due to difficulty in handling and the ease of inducing stress in these species. With the growing incidence of respiratory disease in bison and deer, tulathromycin is frequently used in an extra-label manner because no antimicrobial drugs are registered or approved in North America for use in these species. Significant interspecies differences in pharmacokinetic processes and tissue distribution characteristics warrant specific investigation in these species of interest (Toutain et al., 2010). Studies are needed to investigate tulathromycin efficacy and safety in bison and deer in order to effectively treat disease and, since these animals are used for meat, there is also a need to ensure food safety. The primary goals of our research were first, to investigate the pharmacokinetics of tulathromycin in bison and deer, which would provide some information regarding an appropriate dosage regimen in these species. Second, we wanted to investigate tulathromycin distribution and depletion in tissues, which, along with further investigation of the pharmacokinetic profile, will present information regarding withdrawal time of tulathromycin in these species, which is important for consumer safety.

These objectives required the development and validation of a sensitive and accurate LC-MS analytical method for quantification of tulathromycin in bison and deer serum and tissues. Following analytical method development and validation, the pharmacokinetics were investigated in bison and deer serum, and muscle and lung tissues analyzed in deer. Following a single 2.5 mg/kg bw subcutaneous injection of *Draxxin*, tulathromycin demonstrated early maximal serum concentrations, wide distribution, and slow elimination characteristics in these species, predictive for this compound. The mean and standard deviation of each PK parameter estimate from our bison and deer studies compared to cattle and select other species are reported in **Table 6.1**.

**Table 6.1:** Mean and standard deviation (SD) of the pharmacokinetic parameters from our bison and deer studies compared to those in cattle and select other species. All animals received a 2.5 mg/kg bw subcutaneous injection of *Draxxin*, except swine which received a 2.5 mg/kg bw intramuscular injection. All values were obtained in serum or plasma.

PK parameter	Our study 2013 Bison	Our study 2013 Deer	Nowakowski et al., 2004 Cattle	Gäler et al., 2004 Cattle	Benchacoui et al., 2004 Swine	Young et al., 2010 Goats	Scheuch et al., 2007 Foals
	Mean ± SD (n=10)	Mean ± SD (n=10)	Mean ± SD (n=6)	Mean ± SD (n=6)	Mean ± SD (n=6)	Mean ± SD (n=10)	Mean ± SD (n=6)
k (h <sup>-1</sup> )	0.0033 ±0.0006	0.0027 ±0.0009	0.0077 ±0.0012		0.0092 ±0.0016	0.0063 ±0.0017	
AUC <sub>0-inf</sub> (ng*h/mL)	9341 ±5087	4883 ±733	18700 ±1800		15600 ±3150	12500 ±2020	21900 ±3370
C <sub>max</sub> (ng/mL)	195 ±157	359 ±224	500 ±400	300 ±400	637 ±207	633 ±300	410 ±192
t <sub>max</sub> (h)	*4.2 ±4.6	1.3 ±0.2	1.8 ±3.0	0.7 ±3.0	0.3 ±0.0	0.4 ±0.3	3.9 ±3.9
Cl/F (L/h/kg)	0.4 ±0.2	0.5 ±0.1				0.2 ±0.04	
V <sub>d</sub> /F (L/kg)	111 ±66	208 ±52	11			33 ±6	15 ±3
half-life (h)	214 ±43	281 ±86	90	110 ±40	76 ±14	110 ±20	117 ±21
AUMC <sub>0-inf</sub> (ng*h <sup>2</sup> /mL)	1985 ±1132	1087 ±466					
MRT (h)	208 ±40	217 ±77				81 ±20	
*Median = 1.04							

## 6.1 Principal Findings

The bison and deer serum extraction methods were slightly different and demonstrated the importance of developing and validating a method for each specific species and matrix of interest. Acetonitrile was used for protein precipitation in each bison serum sample. However, when acetonitrile was added to each deer serum sample, a coagulated mass was evident, rather than a protein precipitate. After experimentation, sample precipitation with the addition of trifluoroacetic acid produced a solid precipitate, but resulted in extensive precipitate formation and poor sample recovery. We resorted to larger initial deer serum sample volumes to use for precipitation and transferred only half of the supernatant to continue the extraction procedure the same way as done in bison serum. We can hypothesize the presence of endogenous substances in deer serum not present in the bison serum, which resulted in the coagulated mass following acetonitrile precipitation. The serum biochemistry between bison and deer may be different. Species differences in proteins, fatty acids, triglycerides, or chylomicrons, as well as nutritional differences between these species may result in different serum components and thus, the need for different extraction procedures.

The principal finding in bison included a lower mean  $C_{\max}$  (195 ng/mL) compared to cattle (300 to 500 ng/mL) and a much longer half-life (214 hours) compared to cattle (90 to 110 hours) (Gáler et al., 2004; Nowakowski et al., 2004). Tulathromycin AUC and  $C_{\max}$  values were almost two-fold less in bison, suggesting that bioavailability following subcutaneous injection is lower in bison relative to cattle and other species. On the other hand, more rapid elimination kinetics in bison may explain the lower AUC and  $C_{\max}$  values in bison relative to other species. The longer half-life of tulathromycin in bison might be explained by larger volume of distribution; however, the concentration-time profiles in bison may represent flip-flop kinetics. Absorption kinetics may become the rate limiting process governing the terminal natural logarithmic concentration-time profiles with slow release kinetics of tulathromycin from the subcutaneous injection sites. Tissue distribution studies to confirm extensive distribution of tulathromycin in lung tissue and clinical efficacy studies are needed to help determine whether the 2.5 mg/kg bw subcutaneous dose is adequate for use in bison.

In deer, the mean  $C_{\max}$  (359 ng/mL) is comparable to those reported in cattle (300 to 500 ng/mL) and the half-life (281 hours) was also much longer compared to cattle (90 to 110 hours)

(Gáler et al., 2004; Nowakowski et al., 2004). The similar  $C_{\max}$  value compared to cattle and other species suggests comparable bioavailability of tulathromycin. The elevated half-life is likely due to extensive tissue distribution. Tulathromycin was extensively distributed to lung ( $C_{\max} = 4657$  ng/g) and muscle ( $C_{\max} = 2263$  ng/g) in deer, and the highest tissue levels were reached within 7 to 14 days (first and second sampling time points) after injection. Drug concentrations in the tissues were still detected 56 days after treatment demonstrating slow elimination. This characteristic is supportive for the administration of tulathromycin as a single injection therapy, resulting in prolonged potential exposure of tulathromycin to bacterial pathogens associated with respiratory disease of deer. When considering food safety, the use of tulathromycin in deer seemingly requires a much longer withdrawal time interval (perhaps greater than 56 days) in comparison to the times established for cattle (44 days). Future studies are necessary to determine an accurate withdrawal interval of tulathromycin in deer species.

Tulathromycin had a high degree of pharmacokinetic variability between individuals of the same species. Many reasons explain the commonly observed inter- and intra-individual variation and differences in drug pharmacokinetics. In general, macrolides tend to exhibit significant variation in plasma concentrations because of their high volumes of distribution (Benchouai et al., 2004; Nowakowski et al., 2004; Zhanel et al., 2001). Furthermore, reasons for the variability noted between individuals in the present study may be due to differences in bioavailability. Both bison and deer species received a 2.5 mg/kg bw subcutaneous injection of tulathromycin. The difficulty with handling these species makes injections difficult and some animals may have received only a partial dose or an intramuscular deposition occurred with dose administration, rather than injection into the subcutaneous tissue. Rate and extent of absorption is known to vary quite significantly between subcutaneous and intramuscular injections for some drugs. *Draxxin* is also designed as a slow-release formulation, and differences in the physiological environment of the drug depot site can lead to differences in the rate of release and absorption from the site. In addition, these are supposed to be healthy animals, but any underlying physiological condition can affect the absorption, distribution, and clearance of a drug. As with humans, genetic factors could also contribute to PK differences.

When we assess the clinical importance of this interindividual variation, we know some animals will fail to respond adequately to tulathromycin. Every drug has a therapeutic range for effectiveness and when a drug is below that range (low  $C_{\max}$ ), the drug will be ineffective for

most individuals in a population. Alternatively, a drug above the range may be toxic. We make suggestions and choose a dosage regimen based on what works for the majority of animals. When it comes to choosing a recommended withdrawal period for a drug with high pharmacokinetic variation among individuals, the withdrawal time will be extended (for the 99<sup>th</sup> percentile of the slowest depleting population, with a 95% confidence interval) so as to address the safety issue for those eating animal derived products. While more study is needed to establish a recommended withdrawal time, the long half-life of the drug observed in deer in both serum and tissues suggests a withdrawal period beyond 56 days and the extensive interindividual variation suggests an even longer withdrawal period to account for variability among individual animals.

Using LC-MS analytical methodology and pharmacokinetic analysis together, we were able to provide information related to a dosage regimen design in both bison and white-tailed deer. This information is very valuable to veterinarians and producers of these species who may suffer significant losses each year due to respiratory disease. Pharmacokinetic analysis suggests the bioavailability of tulathromycin may be adequate in deer, but not in bison. Deer muscle tissue analysis provides veterinarians and producers with important information regarding the withdrawal time of tulathromycin. Tulathromycin in muscle was still detected 56 days after treatment, much longer than the withdrawal period in cattle (44 days), suggesting the withdrawal period in deer will be longer than cattle. While the data obtained may be of significance, tissue distribution and depletion studies are needed to further assess these recommendations.

## **6.2 Study Limitations**

The pharmacokinetic profile of tulathromycin was investigated in bison and deer, and a method was developed for quantification of tulathromycin in tissues of these animals. Lung and muscle tissues for the white-tailed deer tissue depletion study were also analyzed; however, due to time constraints, the remaining liver, kidney, and adipose tissues were not analyzed. This will be a focus of future studies. Although a bison tissue depletion study is necessary to understand withdrawal times in this species, such a study will be very costly and significant logistical concerns exist with respect to how to handle the animals between euthanasia and tissue collection and with carcass disposal.

The marker residue for tulathromycin in bovine and swine has been established as the hydrolytic fragment of tulathromycin (CP-60,300) (Health Canada-2, 2013; EMEA, 2004). However, we chose to analyze the parent compound of tulathromycin (CP-472,295). The marker residue is required for regulatory purposes. The focus of our study was the pharmacokinetics of tulathromycin in bison and deer species, which requires investigation of the active compound (parent tulathromycin). Therefore, it was important to assess the parent compound concentration in tissues, in order to put the values into context with the serum. In addition, one may question why this metabolic fragment has been chosen as the MR, since the metabolism of tulathromycin is very minimal (10%). Nonetheless, the analysis of parent tulathromycin for the present study was important for investigating the pharmacokinetics of tulathromycin in bison and deer species.

One overall goal for the assessment of tulathromycin in bison and deer is to be able to identify appropriate dosage regimens in order to assure a more effective therapy treatment for these species. Pharmacokinetics and PK research will provide information about appropriate dose and frequency of administration, however this is only one part of the requirement. Pharmacodynamic (what the drug does to the body) assessments are also important for a complete understanding of drug efficacy (Evans, 2005; Zhanel et al., 2001). In order to provide an appropriate dosage regimen for tulathromycin use in these species, the pharmacodynamics of this drug also requires assessment. Studies such as MIC for tulathromycin for respiratory pathogens found in bison and deer will need to be determined. It will also be important to assess this antibiotic in animals with respiratory illness. Nonetheless, pharmacokinetic assessment is an important starting point for providing an effective treatment for tulathromycin in bison and deer.

### **6.3 Future Directions**

Future studies will focus on analyzing the remaining tissues, including liver, kidney, and adipose in deer for a more complete tissue distribution and depletion assessment. In the deer tissue pilot study, we identified a need for analyzing an earlier time point in tissues to assess peak concentrations following subcutaneous injection of tulathromycin. This information is important in assessing dosage regimen efficacy for tulathromycin in deer. As such, future assessments should consider incorporation of earlier collection time points after dosing, particularly at 24 hours for comparison in cattle and other species. We also determined



tulathromycin levels remain detectable 56 days after treatment, which is a longer time period than the established withdrawal time in cattle (44 days). This suggests the need to incorporate later collection time points after dosing in order to ensure when the drug is virtually eliminated from all tissues.

Tissue distribution studies are important in bison to gain a better understanding of tulathromycin pharmacokinetics.  $C_{\max}$  was lower in bison compared to cattle, but without tissue data, we are unable to assess if this is due to poor bioavailability or a greater penetration into tissues, particularly the lung. Tissue distribution studies, to confirm extensive distribution of tulathromycin in lung tissue, and clinical efficacy studies are needed to determine whether the 2.5 mg/kg bw subcutaneous dose is adequate for use in bison. Lung tissue distribution studies will help determine the clinical efficacy of tulathromycin in bison.

Both pharmacokinetic and pharmacodynamic parameters are important for the determination of drug efficacy. Pharmacokinetic evaluation in bison and deer provided an important piece for establishing a dosage regimen in these species. Future studies are required to assess the pharmacodynamics of tulathromycin in bison and deer in order to identify an adequate species-specific dosage regimen in bison and deer. The MIC for tulathromycin against respiratory pathogens associated with each species will determine what concentration is needed to effectively kill bacteria. Supportive efficacy trials of tulathromycin in infected animals are also necessary to confirm whether a dosage regimen recommended for cattle will produce efficacious therapeutic outcomes in bison and deer for the treatment and prevention of respiratory disease.

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